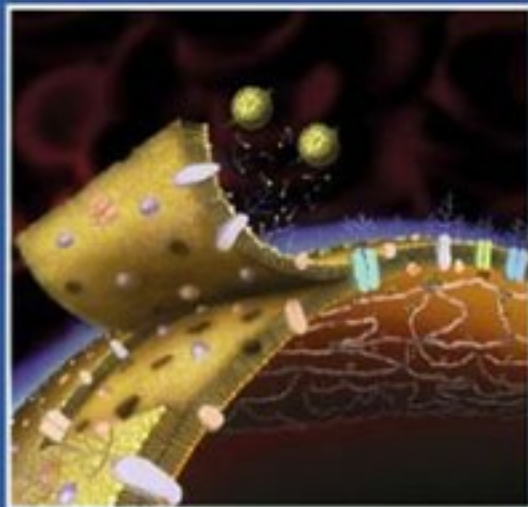


Yoshihito Yawata

Cell Membrane

The Red Blood Cell as a Model



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Preface

I am pleased to have had the opportunity to present an overview of red cell membranes in normal and disease states with my background of nearly 30 years in this area of research.

I believe that this kind of publication on red cell membrane is a very timely summary of all the results obtained by the tremendous efforts worldwide by all of the scientists in this field during the past few decades.

As reviewed in Chapter 1, the general concepts of red cell membrane abnormalities and the categories of each red cell membrane disorder are now well established. Clinical and biochemical analyses of these abnormalities were nearly completed in the 1980s, and most of their genotypes have also been disclosed in the 1990s. Thus, we are able to obtain a perspective view of these disorders.

However, it is also true that we have actually studied the genomic mutations *per se* of determined red cell membrane protein genes at one pole, and also the protein abnormalities in peripheral red cells at another pole. Thus, we have to realize that only some parts of the steps between genomes and proteins have been clarified. Postgenomic investigations will become crucial to elucidate the pathogenesis of red cell membrane disorders in the future, that is, genetic and epigenetic modification, the expression of mRNA, protein synthesis in the Golgi apparatus, protein precursors and their isoforms, trafficking of proteins in cytoplasm in the cell, incorporation of preformed proteins into the stereotactic ultrastructure, functions with these membrane proteins under the environment of the lipid bilayer.

Therefore, we should carefully evaluate the results obtained in the genotype and take the peer look on the scientific achievements in the phenotype. We have to revisit many of the wonderful papers which have been published.

I started my academic career in hematology at the Third Department of Internal Medicine, the University of Tokyo in 1963 after finishing clinical training of three years including internship there. My research topics were storage iron metabolism and glycolytic enzymology, especially glutathione metabolism (directed by Professor Kiku Nakao and Dr. Masao Hattori).

I extended my research on glutathione reductase at the UCLA Harbor Campus in Hematology (Director: Professor Kouichi, R. Tanaka) in 1969–1971. In 1970, a breath-taking procedure at that time was published, that is, the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), which enabled red cell mem-

brane proteins to be solubilized completely. I decided to move to the University of Minnesota Medical School (Hematology), where Professor Harry S. Jacob postulated the presence of abnormalities of membrane proteins in red cell membrane disorders (see Chapter 1). I initiated studies of a possible role of cyclic nucleotides for red cell membrane protein function (1971–1974) in addition to research on hyperalimentation hypophosphatemia and uremic hemolysis.

In 1974, I was promoted to Associate Professor of Medicine, soon after to Professor of Medicine (Hematology), Chief, Division of Hematology, Kawasaki Medical School, Kurashiki City, Japan, where Professor Susumu Shibata, the famed scientist in hemoglobinopathy research, was the Director. I immediately started to set up my own laboratory for red cell membrane research and prepared a standard screening protocol (see Chapter 9). Since that time, I devoted myself for 24 years to membrane research until my mandatory retirement in 2001.

I was so pleased to have been at the Kawasaki Medical School, where research circumstances were excellent, with seven independent research centers, especially the Research Centers for Biochemistry (I was the Director in 1996–2001), for Electron Microscopy, and for Immunology and Cell Culture. These research facilities were so helpful for my membrane studies. I was also supported by research grants from the Kawasaki Medical School continuously.

My laboratory has been the reference laboratory assigned to the Research Committee for Hemolytic Anemias and further for Idiopathic Disorders of Hematopoietic Organs from the Japanese Ministry of Health and Welfare of the Japanese Government. I greatly appreciate the nationwide assistance for my research for red cell membranes. The extensive scientific support by Grants-inAid for Scientific Research and International Research Program: Joint Research from the Ministry of Education Science, Sports and Culture of the Japanese Government should also be mentioned. From this background, I have had the opportunity to study 1014 patients of 605 families with red cell membrane disorders in Japan.

From these standpoints, this book is aimed to review the present status in red cell membrane research in normal and disease states. For this purpose, my own experience in cases with red cell membrane disorders are widely utilized, with many electron micrographs and figures being provided for comprehensive understanding. I would like to express my sincere appreciation to the many scientists who gave me their permission to use their original figures and tables, which were previously published in their articles.

I do hope that this book will help readers to appreciate the achievements in this field of science, which were obtained by timeless efforts by investigators with their tears and joy, and to guide the research projects into the future. This was the major rationale why I decided to start writing this monograph.

Finally, I would greatly express my sincere appreciation to Professor Samuel E. Lux, IV, who was graceful enough to write a forward for this book. He is a most respected and distinguished scientist with tremendous knowledge and experience in this field.

January 27, 2003 Kurashiki, Japan
Yoshihito Yawata, M. D., Ph. D.

Foreword

The erythrocyte membrane is less than 0.1 % of the cell's thickness and only about 1 % of its weight, but it is important. It sequesters glutathione and other compounds required to keep hemoglobin reduced and functional, and selectively retains vital metabolites, while allowing metabolic debris to escape. It perfectly balances cation and water concentrations so that red cells do not shrink or burst, and simultaneously exchanges tremendous numbers of bicarbonate and chloride anions, which aid transfer of carbon dioxide from the tissues to the lungs. It fashions a slippery exterior so that red cells cannot adhere to each other or to vessel walls and clog capillaries. Finally, buttressed on the inside by the "membrane skeleton", a geodesic-like protein structure, the erythrocyte membrane achieves the critical combination of strength and flexibility needed to survive for four months in the circulation. Failure of any of these, or numerous other functions, shortens red cell survival, and leads to disease.

Professor Yoshihito Yawata examines all aspects of the erythrocyte membrane in this book, which is, I believe, the first book devoted solely to this important structure. There is a special emphasis on the red cell membrane skeleton and membrane diseases. These are areas in which Prof. Yawata is particularly expert. He was first introduced to red cell membranes in the early 1970's, during his training with Dr. Harry S. Jacob in Minnesota. Following his return to Kawasaki Medical School, he established a laboratory devoted to the study of red cell membrane diseases and soon became a national referral center and a Japanese government-assigned reference institute.

Prof. Yawata was Professor of Medicine at Kawasaki Medical School, and Chief of the Division of Hematology until 2001. He is now Professor Emeritus. He served two terms as Director of the Japanese Society of Hematology and was President of the Japanese Society of Clinical Hematology in 1999–2000, a high honor. Prof. Yawata has been greatly aided in his membrane work by his lovely wife, Dr. Ayumi Yawata, who is an expert electron microscopist, and whose wonderful pictures appear throughout the book.

During his career, Prof. Yawata and his colleagues have studied more than 1000 patients with red cell membrane diseases, probably more than any other laboratory in the world. This book is his *magnum opus*, the summation of his life's work, and will be a invaluable resource for all of us who are interested in the red cell.

Samuel E. Lux IV MD
Robert A. Stranahan Professor of Pediatrics
Harvard Medical School
Chief, Division of Hematology/Oncology
Children's Hospital Boston

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I would like to express my sincere appreciation to my colleagues in the Division of Hematology, Kawasaki Medical School, who are listed below, especially to Dr. Akio Kanzaki for his excellent biochemical and genetic contributions, and for his timeless effort, and to Drs. Osamu Yamada, Kosuke Miyashima, and Takemi Otsuki for their academic and personal advice and encouragement.

The many fruitful collaborations with foreign scientists in this field should also be mentioned; (1) Professor Jean Delaunay (Faculté de Médecine Grange-Blanche in Lyon, France) under the auspices of the Japan Society for Promotion of Science (JSPS)-Centre Nationale de la Recherche Scientifique (CNRS)-Japan/France Cooperative Joint Research Program (1992, 1994), which was further extended as the Monbusho's International Scientific Research Program: Joint Research (Nos. 0604421, 07457236, and 08044328: 1994, 1997); (2) Professor Walter Dörfler (Institut für Genetik, Universität zu Köln) under the auspices of the Monbusho's International Scientific Research Program: Joint Research (Nos. 09044346, 09470235, 10044329, 12470206, and 14370311: 1997 to the present day); (3) Professor Stefan Eber (Georg-August-Universität Göttingen) under the JSPS-Deutsche Forschungsgemeinschaft (DFG) Cooperative Joint Research Program (1997, 1999), and unofficial collaborations with Professors Jiri Palek and Carl M. Cohen in Boston, Samuel E. Lux in Boston, Bernard Forget and Patrick Gallagher at Yale, Stephen B. Shohet in San Francisco, Harry S. Jacob in Minneapolis, Kouichi R. Tanaka in Los Angeles, and many others.

Scientific support was also obtained for many years from research grants for Idiopathic Disorders of Hematopoietic Organs from the Japanese Ministry of Health, Welfare, and Labors (1974–2002), and from the Kawasaki Medical School (1975–2001).

I am greatly indebted to Dr. Andrea Pillmann, Karin Dembowsky, and Hans-Jochen Schmitt from Wiley-VCH for their cordial help and encouragement in editing and producing this book, and to Ms. Tomoko Yamamoto and Kyoko Sato for their tremendous secretarial assistance without which this timely publication would have been absolutely impossible.

Finally, I would like to express my heartfelt appreciation to my wife Ayumi Yawata, M. D., Ph. D., for her tremendous contributions in the field of molecular electron microscopy, which are clearly visible everywhere in this book, and for her warm and genuine support throughout my life.

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Institutes and hospitals from which red cell membrane disorders have been consulted.

January 27, 2003 Kurashiki, Japan
Yoshihito Yawata, M. D., Ph. D.

1

Introduction: History of Red Cell Membrane Research

1.1

Invention of Optical Microscopes and Their Application to Hematology

To identify abnormalities in blood cells as the pathogenesis of blood diseases, recognition of these blood cells is an absolute prerequisite. For this purpose, invention of instruments to identify such small blood corpuscles, which could not be seen with the naked eye, was definitely to be expected. The history of the development of the light microscope is also a history of hematology, and particularly of blood cytology [1, 2].

It was Roger Bacon (1214–1294) in England who first discovered the fact that lenses could magnify small particles. Three hundred years later, new technology made the production of optical lenses of a higher magnification possible. Zacharias Janssen invented an optical microscope, and Robert Hooke followed on from this work. The person who introduced light microscopy into medical science was Athanasius Kircher, from Germany, in 1657.

Jan Swammerdam (1637–1680) in Amsterdam first identified red cells with a light microscope, and he described them as “ruddy globules”. Anton van Leeuwenhoek (1632–1723) in Delft, Holland performed observations of various blood cells with a light microscope equipped with his own lenses (with from $\times 40$ to $\times 275$ magnification, and $1.4\ \mu$ resolution). This work was published in the *Philosophical Transactions of the Royal Society of London* in 1674, unfortunately with little public attention.

After this period, scientific achievements were made in establishing a method for dry smear preparations of blood cells in peripheral blood, and staining methods for these blood cells. Paul Ehrlich (1854–1915) in Silesia, Germany and a pathologist, Rudolf Virchow (1821–1902) from Pomerania, Germany, made great contributions to these achievements. The staining methods initially invented by Ehrlich were later developed further into more sophisticated procedures, by Romanowsky, Giemsa, Wright, and many others, and these are now widely utilized. Two major optical companies in Germany, Zeiss and Leitz, started delivering their excellent light microscopes for medical applications in 1851. Zeiss, in Jena, Germany, was established in 1846, and, 20 years later, had already delivered more than 1000 microscopes, which had been newly designed by Abbe in Jena, to scientific laboratories. The Leitz company, which was initially established as the Karl Kellner

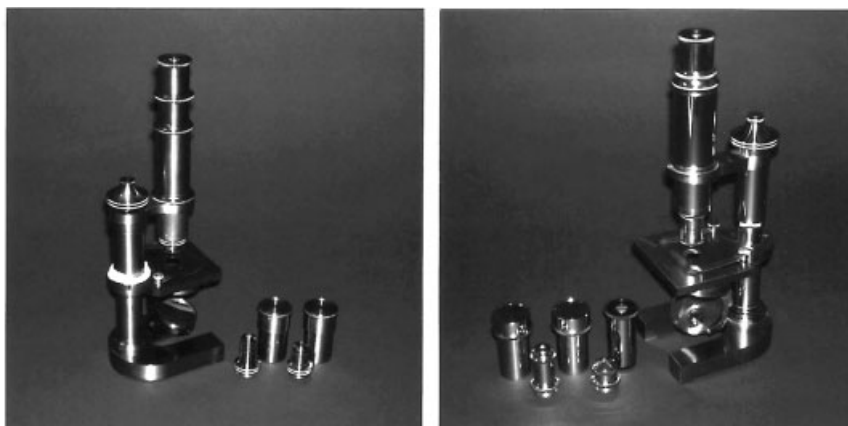


Figure 1.1 Light microscopes. Brass replicas made by Zeiss (in 1880) on the left, and by Leitz (in 1899) on the right.

Optisches Institut in 1849, delivered the first microscope with an excellent achromatic lens in 1851. The number of microscopes delivered had reached more than 50 000 by 1899. The author is proud to own replicas of microscopes manufactured by Zeiss (in 1880) and Leitz (in 1899), which are beautifully made in brass (Fig. 1.1).

Methods for counting cells were also developed by utilizing these light microscopes, in particular by Karl Vierordt in Tübingen in 1852, who estimated the number of red cells in peripheral blood to be $5.174 \times 10^6 \text{ mm}^{-3}$. This value is respectably close to the actual number determined by current advanced electronic methods of measurement. In addition, in 1910 Cecil Price-Jones, in England, published his work on the distribution pattern of peripheral red cells of various sizes, the so-called “Price-Jones curve”.

With this background, the opportunities to elucidate the presence of hereditary spherocytosis as an abnormality of red cell morphology by light microscopy blossomed.

1.2

Discovery of Hereditary Spherocytosis by Light Microscopy

In 1871, Vanlair and Masius in Liège, Belgium encountered a family where the mother and her daughters suffered from familial jaundice with splenomegaly. They found that the red cells of these patients were small and spheroid (4 μm in diameter) under a light microscope and reported these morphological observations as “de la microcythémie” at the Belgium Royal Academy of Medicine (Fig. 1.2). This is believed to be the first report of hereditary spherocytosis [3]. They also mentioned that the pathogenesis of increased hemolysis lies in “globules atrophiques”.

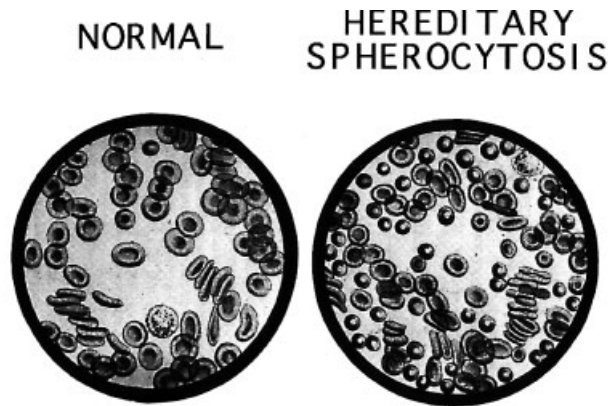


Figure 1.2 A lithograph of de la microcythémie published by Vanlair and Masius in 1871.

Wilson and Stanley (1893) in England found a hereditary disorder with chronic anemia, splenomegaly, jaundice and gallstone episodes, and further mentioned that red cells were packed into the patient's spleen [4]. Unfortunately, they did not mention any morphological characteristics of the red cells in this disorder. Le Gendre (1897) [5] and Hayem (1898) [6] also described acholuric jaundice without increased plasma bilirubin or increased urinary bilirubin excretion.

Oskar Minkowski (1900) in Germany reported on eight patients with “hereditärer chronischer Ikterus” in Alsace, who had suffered from life-long jaundice and marked splenomegaly [7].

Anatole Chauffard (1907) in France made enormous contributions to the understanding of the pathophysiology of the red cell abnormalities present in hereditary spherocytosis [8]. He found a 24-year-old man with congenital jaundice, severe anemia, gallstone episodes, and urobilinuria (l'ictère congenital de l'adulte). He examined the family of this patient by utilizing the osmotic fragility test, which was invented by Vaquez, and found a markedly increased osmotic fragility in the red cells of this family. In addition, he also noticed that their red cells were small in diameter (5.89 μm on average: 7.5–4.0 μm) indicating the presence of microcytosis. These smaller red cells were much more osmotically fragile than normal-sized red cells. In his description, the functional abnormalities of the patient's red cell membranes were clearly demonstrated.

Gänsslen (1922) reported on “hämolytischer Ikterus” with excellent clinical descriptions of exacerbation factors for hemolysis (common cold, infection, menstruation, pregnancy, etc), erythroid hyperplasia in bone marrow, autosomal dominant inheritance, and so on [9]. He classified this disorder into three categories, i. e.: (1) klassische (polysymptomatische) Form, (2) oligo-oder-monosymptomatische Form, and (3) kompensierte Form (without jaundice or anemia). Surprisingly, he also raised the possibility of the presence of sporadic cases due to a *de novo* mutation, and the efficacy of splenectomy. In his description, the two major pathogeneses for hereditary spherocytosis were clearly discussed, that is: (1) the presence of spherocytosis (“Kugelform”), and (2) the contribution of the

spleen. Thus, the basic recognition of these pathognomonic mechanisms in hereditary spherocytosis was already well established in the 1920s.

Although the detailed clinical description and the effectiveness of splenectomy in hereditary spherocytosis were clearly known, several decades were required before the pathophysiology of this disorder were understood and genetic abnormalities elucidated. This was not until the development of red cell membrane research as a basic science had been achieved.

1.3

The Dawn of Red Cell Membrane Research

As mentioned previously, Chauffard (1907) and other scientists in Europe actually initiated the elucidation of the pathophysiology of red cell abnormalities in hereditary spherocytosis. However, Castle et al. (1937), of the American school, also made significant contributions to this field of research [10]. They discovered that the membrane surface/cell volume ratio was reduced concurrently with loss of the discoid form of these red cells with hereditary spherocytosis. Ham et al. (1940) pointed out that the increased propensity for hemolysis in the patient's red cells was dependent not on the increased red cell volume but on the decreased effective cell surface of these abnormal red cells [11]. Eric Ponder made a tremendous contribution by clarifying the hemolytic phenomenon of red cells from the biophysical standpoint. He published his renowned monograph of *The Mammalian Red Cell and the Properties of Hemolytic Systems* in 1934 and then in a revised form *Hemolysis and Related Phenomena* in 1948 [12].

John V. Dacie et al. (1954) in the United Kingdom discovered that the red cells found in cases of hereditary spherocytosis were initially swollen, then normalized, and then became further shrunken during their *in vitro* incubation. They also observed a decreased intracellular potassium content with an increased intracellular sodium content in these red cells, and confirmed the results previously reported by Castle et al. that the increased osmotic fragility in hereditary spherocytosis red cells was due to the decreased membrane surface/cell volume ratio. Dacie's monographs *The Haemolytic Anaemias*, which were published in 1954, 1960, and 1985, are excellent publications based on the enormous accumulation of his extensive studies [13–15].

The observations made during this period directed scientists' attention toward the abnormalities of membrane transport in hereditary spherocytosis red cells. Jacob et al. (1964) reported decreased osmotic resistance, increased sodium influx, and a compensatory increase in glycolysis in these red cells, and proposed the theory that the basic pathogenesis of hereditary spherocytosis lies in the "leaky membrane" of these red cells [16]. At this point, a possible causal pathogenesis of hereditary spherocytosis as a red cell membrane disorder was formally proposed.

During this time period, Pranker, as a red cell enzymologist, claimed that increased glycolysis was the pathogenesis of hereditary spherocytosis, but Lawrence Young and his school in Rochester reported that increased glycolysis was merely

one epiphenomena in these hereditary spherocytosis red cells, and was first to clarify that the essential point lies in microfragmentation of the red cell membranes.

The processes of microspherocytosis in hereditary spherocytosis were studied extensively by Marcel Bessis et al. in France. There are generally two pathways for the production of spherocytosis in red cells: (1) an echinocytic pathway which is energy-independent, and (2) a stomatocytic pathway which is energy-dependent. The former appears to be related to exocytosis, and the latter to endocytosis. For these studies, light microscopy with a phase contrast apparatus, and the newly-introduced scanning electron microscope were utilized extensively. Robert Weed and Claude Reed in Rochester collaborated with the Bessis group to publish their own journal *Blood Cells* (1973) [17]. *Red Cell Rheology* was published in 1978 [18] in memory of Weed, who suffered an unexpected early death. With Bessis, Narla Mohandas invented the ektacytometer to determine red cell deformability, and opened up a new field of red cell rheology.

A tremendous biochemical contribution to red cell morphology should be mentioned. Makoto Nakao and his colleagues discovered that red cell shape changes are dependent on the energy of adenosine triphosphate (ATP). With their publication in *Nature* [19], this area of investigation was encouraged extensively and actually developed significantly. It was made clear that adenosine triphosphate (ATP), adenosine triphosphatase (ATPase), and calcium are critical modification factors for red cell deformability. As will be described later in detail, studies on red cell rheology made rapid progress in association with extensive developments on membrane lipid research. La Celle, who followed Weed in Rochester, invented his own apparatus with micropipettes to examine the rheological properties of normal and abnormal red cells. During this period, the name of Jiri Palek, who was born in Czechoslovakia, began to appear in the literature on red cell membrane research [20]. It became the general consensus that red cell deformability is dependent on the level of intracellular ATP, and that, when the ATP content is decreased, increased free calcium is bound to membrane proteins, resulting in increased rigidity of red cell membranes.

Whatever the exact mechanism is, the rigid red cells are trapped, sequestered, and destroyed in the spleen. For this hemolytic event, two mechanisms of increased hemolysis were proposed, that is: (1) auto-hemolysis by the abnormal red cells *per se*, and (2) phagocytosis of these abnormal red cells by macrophages in the spleen. These mechanisms were studied extensively and in particular by the Rochester school (Young, Weed, La Celle, et al.) [21].

At the same time, the mechanism for microspherocyte formation was studied by utilizing echinocytogenic and stomatocytogenic compounds and drugs, especially by Schrier et al. at Stanford University. Deuticke et al. (1968) identified the major determinants for red cell shape to be (1) electric charge, and (2) differences between intracellular and extracellular pHs [22]. Schrier et al. discussed changes in red cell shape from their standpoint of asymmetry of the membrane lipid bilayer by utilizing amphipathic compounds such as chlorpromazine [23].

From these steps in the development of membrane research, it became evident that the pathogenesis of hereditary spherocytosis appeared to be related to possible

abnormalities in the red cell membrane constituents. It is well known that red cell membranes are composed mainly of membrane proteins and membrane lipids. Although at this point, around the early 1960s, membrane proteins had been only partially solubilized, it had already become possible to analyze membrane lipids completely, in about 1957.

The determinations of red cell membrane lipids were initiated by Folch et al. (1957), and Erickson (1958). Pennell described red cell membrane lipid composition in a chapter on normal red cell composition with Table 3 in the monograph *The Red Blood Cell* (edited by Bishop and Surgenor) in 1964 [24]. Van Deenen also discussed the dynamic aspects of red cell membrane lipids in the same monograph [25]. The findings on membrane lipids published by Reed et al. (1960), and Way (1967) are definitely consistent. *Seminars in Hematology* published special issues on *The Red Cell Membrane* by three invited guest editors, R. I. Weed, E. R. Jaffé, and P. A. Miescher, in which analyses of normal red cell membrane lipids were described. Excellent studies on the modifying factors of membrane lipids (diet, aging, blood cell preservation, hepatic dysfunction, abnormal lipoprotein disorders, and hereditary spherocytosis) were also performed by R. Cooper et al. in 1970 [26].

Regarding red cell membrane lipids, it was already known that the total red cell lipid content was approximately 5.00×10^{-10} mg/cell. Approximately 60 % of the total content was phospholipids, 30 % was free cholesterol, and the remainder was mainly glycolipids. The phospholipids were also known to consist of subpopulations of sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine of approximately 25 % each, phosphatidylserine of approximately 15 %, and small amounts of phosphatidylinositol. The presence of asymmetrical distribution of these phospholipids in the membrane lipid bilayers, that is, phosphatidylcholine and sphingomyelin, were chiefly distributed on the outer leaflet, and phosphatidylethanolamine and phosphatidylserine on the inner leaflet, was clarified by Marinetti et al. (1974) [27], and Marfey et al. (1975) [28]. It was also claimed that approximately 37 % of phosphatidylserine was cross-linked to membrane proteins at the inter-molecular distance of 9 Å.

Cooper's excellent review (1970) demonstrated that the red cell membrane lipids in hereditary spherocytosis were essentially normal in unsplenectomized patients, but clearly diminished after splenectomy by 15–20 % compared with those in normal controls [26].

By 1970, studies on red cell membrane lipid anomalies of hereditary origin, such as α -lipoprotein deficiency (Tangier disease) by Fredrickson et al. (1964) [29], and Shacklady et al. (1968) as reviewed by Assmann et al. [30], β -lipoprotein deficiency (acanthocytosis) by Ways et al. (1963) [31], congenital lecithin: cholesterol acyltransferase (LCAT) deficiency by Gjone et al. (1968) [32], and also hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA) by Shohet et al. (1971) [33] had been completed. Shohet clarified the biochemical relationship between plasma lipids and red cell membrane lipids (1972) [34]. Acquired red cell membrane lipid abnormalities were investigated energetically by Cooper et al. [26, 35], specifically with regard to the role of free cholesterol in hepatic disorders, such as spur cell anemia and target cells.

Studies on cationic transport in red cell membranes were initiated by Eric Ponder et al., as described previously, and further extended by Skou, Tosteson, Passow, Parker, and particularly Hoffman. On the clinical hematology side, the topics of “hereditary stomatocytosis (hydrocytosis and desiccytosis)” by Nathan and Shohet were dealt with in a special issue *Red Cell Membrane* in *Seminars in Hematology* as early as 1970 [36].

Despite these glorious advances in research on membrane lipids and ion transport, the most striking characteristic of this third stage was a total lack of knowledge of red cell membrane proteins. In a special issue of *Seminars in Hematology* in 1970, Maddy presented his review article on red cell membrane proteins [37]. Weed, as one of the guest editors, mentioned in his introduction [38] that “Dr. Maddy has aptly summarized the analytical difficulties which underlie the limited amount of work done in this field. This particular field very likely will ultimately prove to be the most rewarding, as well as the most challenging, approach for understanding normal and disease membranes”. At this point, the only analytical methods available were those using cholate, Triton X-100, butanol, phenol, urea, etc., none of which completely solubilized the red cell membranes. However, it is very interesting to note that Maddy mentioned briefly the potential possibility of utilizing sodium dodecyl sulfate (SDS) as a detergent for total membrane protein solubilization in his article. Polyacrylamide gel electrophoresis with this detergent, SDS, was a discovery that led to significant advances in protein chemistry. The first paper on this subject appeared in 1970, the same year that the special issue of *Seminars in Hematology* was published. At the same time, Harry Jacob [39] postulated that contractile proteins, such as the microtubules or microfilaments in muscle cells, should exist in human red cells, and that a possible pathogenesis of hereditary spherocytosis could lie in abnormalities of these contractile proteins in the red cells.

1.4

Commencement of Membrane Protein Biochemistry: Introduction of Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis

At last, the door opened, regarding the analyses of membrane proteins, which had long been a bottleneck in red cell membrane research. Following the development of methods for preparing red cell membrane ghosts by Dodge et al. [40], two brilliant papers were published by Laemmli (1970) [41] and Fairbanks et al. (1971) [42], which definitely promised breathtaking progress in membrane research.

With the method of Fairbanks, red cell membrane ghost proteins could be solubilized completely by sodium dodecyl sulfate (SDS) after their extraction at low ionic strength, and were then subjected to polyacrylamide gel electrophoresis (PAGE). Numerous membrane proteins were separated and identified. The names of these membrane proteins were given in an order based on their molecular sizes, that is: band 1 (α -spectrin), band 2 (β -spectrin), band 2.1 (ankyrin), band 3 (anion exchanger 1: AE-1), band 4.1 (protein 4.1), band 4.2 (protein 4.2), band 5 (actin), band 6 (gly-

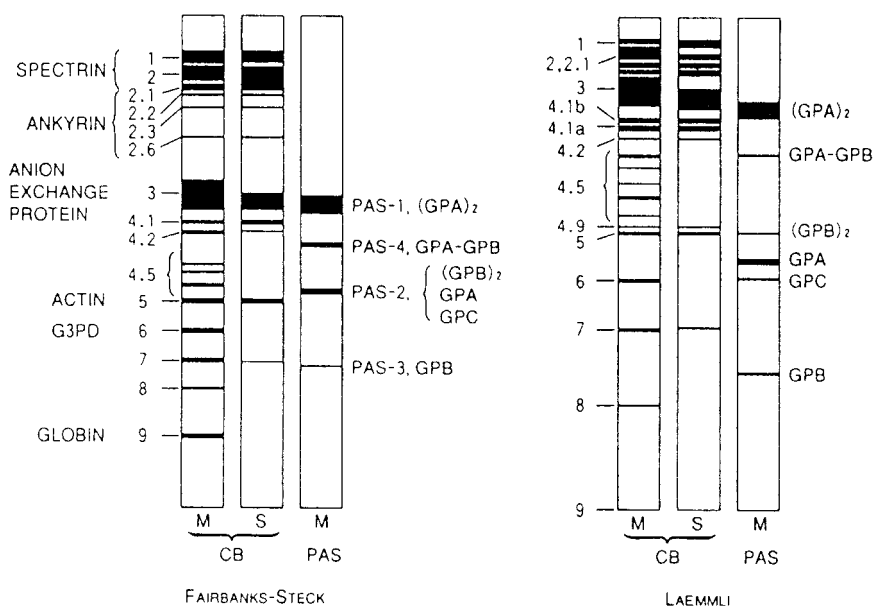


Figure 1.3 A schematic demonstration of the findings of red cell membrane ghost proteins analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) by the methods of Fairbanks and Steck (left), and

Laemmli (right). CB: coomassie blue staining, PAS: periodic acid Schiff staining, M: membrane fraction, S: soluble fraction, GP: glycoproteins, and G3PD: glyceraldehyde 3-phosphate dehydrogenase.

glyceraldehyde-3-phosphate dehydrogenase: G-3-PD), and band 7 (stomatins or aquaporins). For analyses of membrane proteins of smaller molecular sizes, Laemmli's method, with gradient gel electrophoresis, yields better resolution (Fig. 1.3).

Red cell membrane proteins are classified into two groups, that is: (1) peripheral proteins (spectrin, ankyrin, protein 4.1, actin, etc.) and (2) integral proteins (band 3, glycoproteins, etc.). They are also categorized functionally into three groups, that is: (1) cytoskeletal proteins (spectrin, protein 4.1, actin, etc.), (2) integral structural proteins (band 3, glycoproteins, etc.), and anchoring proteins (ankyrin, protein 4.2, etc.) (Table 1.1).

Regarding membrane models, Danielli first began studying cell membranes during the 1930s, and proposed a model for the membrane structure with Davson in 1935 [43]. This is the classical famous Danielli–Davson bilayer model for membrane structure, which is primarily associated with the behavior of lipids in membranes. At first they believed that the proteins were just loosely attached to the two surfaces of the membrane by polar forces, and they also visualized a lipid bilayer more or less covered on both sides with molecules of unfolded globular protein. The contribution of proteins to membrane structure had been recognized by the two Dutch investigators, Gorter and Grendel, some 10 years earlier than Danielli. At that time, more than 65 years ago, far less was known about protein structures than at present.

Table 1.1 Molecular characteristics of major membrane proteins in human red cells.

Band on SDS gel	Protein	Molecular mass (kDa)		Copy number ($\times 10^3/\text{cell}$)	Relative amount of total ghost proteins (%)	Localization on membrane
		On SDS gel	Calculated			
1	α -Spectrin	240	280	242 ± 20	14	SKL
2	β -Spectrin	220	246	242 ± 20	13	SKL
2.1	Ankyrin	210	206	124 ± 11	5	ANC
2.9	α -Adducin	103	81	30	1	SKL
	β -Adducin	97	80	30	1	SKL
3	Anion exchanger 1 (AE-1): band 3	90~100	102	1200	26	INT
4.1	Protein 4.1	80, 78	66	200	5	SKL
4.2	Protein 4.2	72	77	250	5	ANC
4.9	Dematin	48, 52	43, 46	140	1	SKL
	p55	55	53	80	1	SKL
5	β -Actin	43	42	500	6	SKL
	Tropomodulin	43	41	30		SKL
6	Glyceraldehyde-3-phosphate dehydrogenase(G3PD)	35	36	500	5	SKL
7	Stomatin	31	32		4	INT
	Tropomyosin	27, 29	28	70	1	SKL
8	Protein 8	23	22	200	1	SKL
Glycoproteins:						
PAS-1	Glycophorin A	36	14	1000	1.6	INT
PAS-2	Glycophorin C	32	14	150	0.1	INT
PAS-3	Glycophorin B	20	8	150	0.2	INT
	Glycophorin D	23	11	82	0.02	INT
	Glycophorin E	—	6	(not expressed)		

SDS-gel: sodium dodecylsulfate polyacrylamide gel electrophoresis, SKL: skeletal protein, ANC: anchor protein, INT: integral protein.

Around 1966, Singer in San Diego thought that any successful model or theory of membrane structure must provide an explanation for the difference between the molecules of soluble proteins (hemoglobin, etc.) on the one hand and membrane-bound enzymes and receptors on the other. Wallach and Singer independently postulated a new model of protein–lipid architecture. They visualized the proteins as globular and folded up so as to be amphipathic, possessing one hydrophobic and one hydrophilic end, just as membrane lipid molecules do, though of course the proteins would be much larger. The hydrophobic end would be embedded in the interior of the lipid bilayer, in contact with hydrophobic lipid tails, while the hydrophilic end would be ringed with hydrophilic lipid heads and would also project out into the aqueous medium surrounding the membrane. The fundamental structure was compared with that of icebergs (protein) floating in the sea (lipid) [44]. In 1972, this basic iceberg–sea concept was further extended to the Singer–Nicolsons fluid mosaic model [45], which was supported by extensive advances in membrane protein biochemistry and in electron microscopy with regard to the presence of the intramembrane particles shown by Pinto da Silva and Branton in 1970. Sheetz and Singer suggested that in the iceberg–sea model, the spectrin may serve as a sort of reinforcing scaffolding for the red cell membrane, and speculated that rod-like assemblages of spectrin molecules are attached to, and form bridges between, several integral protein molecules in the membrane, even though the cytoskeletal network was not known at that time.

When SDS–polyacrylamide gel electrophoresis became available for membrane protein analyses in 1970, extensive investigations to elucidate the structure and functions of red cell membrane proteins in normal individuals started immediately. The first membrane protein studied was spectrin. The very important initial contribution was made by Marchesi [46]. This was a big surprise when it was published because, at that time, it was the commonly-held belief that red cell membranes only contained a single protein. The protein structures of α -spectrin and β -spectrin were elucidated in detail by many investigators, especially Marchesi et al., Speicher et al., Bennett et al., and Winkelmann et al. The functional characteristics of α - and β -spectrins were also disclosed, as described in the following text.

The structure and functions of ankyrin as an anchoring protein were also elucidated extensively, especially by Bennett et al., and Lux et al. The detailed descriptions are also given in the text.

Band 3 is a unique protein, which has been studied by Tanner et al. in Bristol, Low et al., Jennings et al., Hamasaki et al., and others. Electron microscopic studies on the intramembrane particles have been carried out by Pinto da Silva, Branton and Cohen et al., and others.

Protein 4.1 was rigorously investigated by Conboy, Chasis, Takakuwa, and others along with Mohandas.

Protein 4.2 was first recognized by its deficiency, which was discovered independently by Hayashi et al. and Nozawa et al. The structural and functional characteristics of protein 4.2 were investigated in particular by Carl Cohen, and Cathy Korsgren in Boston. Glycophorins were initially expected to play a crucial role in signal transduction in red cell membrane functions. This expectation was challenged by

Marchesi et al. in the early 1970s [47], because their molecular structure demonstrates typical transmembrane glycoproteins. However, unfortunately, the En (a-) red cells of complete glycophorin A deficiency were not associated with any clinical problems. The functions of glycophorins and their cellular significance still remain to be elucidated at some point in the future.

Steck et al. [48] made the important contributions in devising methods to prepare inside-out and right side-out membrane vesicles, and the use of these vesicles to show that membrane proteins were asymmetrically organized – some inside and some outside.

Other minor proteins, such as adducin, p55, dematin, tropomodulin, tropomyosin and others have also been investigated.

Finally, Lux [49] came up to the first nearly correct model of the red cell membrane skeleton, which clarified what was at the time a very confusing field and led to many additional hypotheses and experiments. This model was indeed the most important contribution to this field by him.

1.5

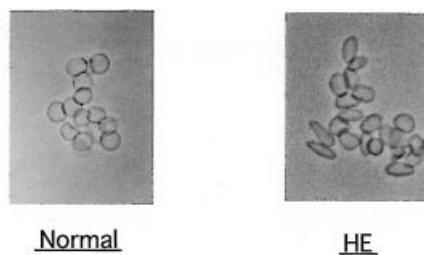
Elucidation of the Pathogenesis of Red Cell Membrane Disorders

In parallel with the investigation of normal red cell membrane protein biochemistry, or a little bit later, the disease states of red cell membranes finally became the targets for investigation around 1980 [50–56].

The first disorder to be examined by molecular biochemistry was hereditary elliptocytosis (HE), because of distinct morphological observations by Lux et al. and Palek et al. They recognized that red cell ghosts themselves remained elliptocytic in their shape, even after they had been prepared from elliptocytes in the peripheral blood of the patients with hereditary elliptocytosis. Therefore, they further prepared Triton shells from these red cell ghosts, and found the critical phenomenon that the morphology of the Triton shells was still elliptocytic (Fig. 1.4). This important finding clearly indicated that the basic molecular defect should exist in their cytoskeletal network, because the Triton shells are basically composed of cytoskeletal proteins.

Shortly after this finding had been made, Palek et al. detected a peptide abnormality at the α I domain in the N-terminal region of α -spectrin by SDS–PAGE and peptide-mapping analysis. This anomaly was named HE [$\text{Sp}\alpha^{1/74}$], because an abnor-

Figure 1.4 The triton shells prepared from red cells of hereditary elliptocytosis (HE) patients still demonstrate elliptocytic shape (right) compared with normal subjects (left) showing discoid morphology.



mal 74 kDa peptide was detected concomitant with the decreased normal 80 kDa peptide of the α I domain of the α -spectrin. The region at the N-terminus of α -spectrin is now known as a functionally critical region for self-association with the C-terminal region of β -spectrin. Following this discovery, many mutations, mostly at the N-terminal region of α -spectrin, were detected, such as HE [Sp $\alpha^{I/74}$], HE [Sp $\alpha^{I/78}$], and HE [Sp $\alpha^{I/65}$]. Ethnic differences were also clarified, i.e.: HE [Sp $\alpha^{I/65}$] and HE [Sp $\alpha^{I/46}$] were predominant in populations of African origin and much less in the Caucasian population; HE [Sp $\alpha^{I/78}$] was only detected in Caucasians; and in Japan, only one trait of HE [Sp $\alpha^{I/74}$] was reported, indicating that α -spectrin anomalies appear to be substantially low in incidence.

β -spectrin anomalies were also detected with truncated abnormal β -spectrin peptides, which were later proven mostly to be due to exon skipping of the C-terminal region of the β -spectrin gene, as discussed in this text in detail.

In some HE patients, severe anemia with significantly increased jaundice was observed. The red cell morphology was strikingly bizarre with marked poikilocytosis and red cell fragmentation. These red cells demonstrated extensive fragmentation when they were heat-treated at 37–48 °C. This condition is known as hereditary pyropoikilocytosis (HPP). Most HPP patients have been proven to be homozygotes or compound heterozygotes of common HE. These findings were later confirmed by gene analyses.

Protein 4.1 deficiency is also pathognomonic for HE. Four independent patients have been proven to be completely protein 4.1-deficient, and other patients (one-third of the Caucasian HE) have been shown to be partially protein 4.1-deficient. Most Japanese HE patients have become known owing to this partial protein 4.1 deficiency.

Investigations of spectrin abnormalities pathognomonic for HE were carried out in the 1980s with great success.

In contrast, studies on hereditary spherocytosis were not initiated essentially until early 1990s, except for one observation in 1985 by Agre et al. [57], who reported significant spectrin deficiency in hereditary spherocytosis of autosomal recessive inheritance. Before this observation, the presence of severe spectrin deficiency was recognized in several strains of mice used as animal models for hereditary spherocytosis. In the homozygous states of sph/sph, ha/ha, and ja/ja, severe spectrin deficiency was noted with striking microspherocytosis (Bernstein et al., 1980) [58].

Among these mice strains, the nb/nb mice also showed a marked spectrin deficiency, but this was associated with the primary defect of ankyrin. Thus, ankyrin deficiency was found to induce a combined deficiency of ankyrin and spectrin. The mouse strain lacked the short arm of the mouse chromosome 8 (8p), which is the site for ankyrin. Several reports of hereditary spherocytosis in human beings noted that it was associated with the lack of human chromosome 8, such as the deletion of 8p11–p21 [59]. The chromosome site of glutathione reductase is known to be present at 8p21.1. In some patients, combined abnormalities of HS and glutathione reductase deficiency were reported. Therefore, ankyrin deficiency was expected to be present in these patients. We also experienced two patients with

exactly this combination. Naturally, ankyrin deficiency was expected, but actually the ankyrin content was not reduced in these two patients. From our present knowledge, it is now clear that the mutated allele of the ankyrin gene was not expressed and that the remaining normal allele compensated by producing the normal ankyrin molecule.

The same situations exist in HS, particularly that of autosomal dominant inheritance. Since 1970, SDS–PAGE has been widely utilized to detect biochemical abnormalities in mutated membrane proteins in patients with red cell membrane disorders, such as mutated membrane proteins with abnormal electrophoretic mobility (such as in β -spectrin anomalies), and decreased amounts of the determined membrane proteins. Although SDS–PAGE is essentially an excellent method and has been used widely, it is still not easy to detect a minimal reduction with it, such as one of –10 to –20 % of the normal values. This difficulty is especially true when making a quantitative determination of the ankyrin content with SDS–PAGE. Ankyrin content is known to be influenced significantly by increased reticulocytosis in patients with increased hemolysis, such as those with hereditary spherocytosis. This is one of the reasons, but certainly not the only reason, why the elucidation of the pathogenesis of HS was greatly delayed until the early 1990s.

Despite these difficulties, Palek and Jarolim in Boston recognized that approximately one-third of HS patients demonstrated partial deficiency of band 3 [60]. Fortunately, band 3 is known to be less masked by increased reticulocytosis than ankyrin in these disease states. They utilized eosin-5-maleimide for detailed quantitative measurement for band 3 content. Jarolim et al. also discovered functional abnormalities of band 3 proteins, such as band 3 Memphis and band 3 Tuscaloosa, in which the protein 4.2 content was also markedly decreased, because the mutated site of band 3 appeared to be a binding site for protein 4.2, as with band 3 Montefiore reported by Rybicki et al.

Palek et al. investigated Southeast Asian ovalocytosis (SAO) in Papua New Guinea, and discovered marked abnormalities in the band 3 molecule [61]. It is interesting to note that the phenotype of SAO is not HS but HE, despite band 3 abnormality. In 1996–1997, the relative incidence of its membrane protein anomalies was disclosed sequentially. Jarolim et al. (1996) [62] reported that among 166 kindred examples of HS, abnormalities in ankyrin and spectrin made up approximately 60 % of HS membrane anomalies, in band 3 23 %, and in protein 4.2 2 %, with 15 % being of unknown origin. Dhermy et al. in France (1997) [63] reported nearly the same results based on their survey of 80 kindred examples of HS. In the Japanese population [64], as compared with the results in Western countries, abnormalities in ankyrin and spectrin made up 30 % of HS membrane anomalies, in band 3 25 %, and in protein 4.2 30 %, and with 15 % of unknown origin.

Regarding protein 4.2 anomalies, the first reports were published independently by Hayashi et al. and Nozawa et al. in Japan. Total deficiency of protein 4.2 is definitely clustered in Japan [65]. To date, we have experienced 34 patients from 20 kindred examples. Only a few patients have been discovered in countries (Tunisia, Portugal, and Italy) other than Japan. Unique variants of protein 4.2 have also been

found in Japan, that is, the protein 4.2 doublet Kobe and protein 4.2 doublet Nagan. The red cell morphology is complex when compared with those of typical microspherocytosis (observed in protein 4.2 Komatsu), ovalostomatocytosis (mostly of the Nippon type), and even stomatocytosis (protein 4.2 doublets) [66]. Partial deficiency of protein 4.2 is mainly due to mutations of band 3 at its binding site to protein 4.2.

Membrane lipid anomalies appear to be rare in worldwide. However, one of the reasons for this scarcity may be the screening systems for the detection of abnormalities of red cell membrane components. Major laboratories in Western countries do not appear to incorporate membrane lipid analyses as routine screening items. As mentioned in this text, we detected a substantial number of patients with hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCA) simply because membrane lipid analyses are one of the routine screening items at our laboratory at the Kawasaki Medical School [64]. Thus, the apparent high incidence of this disorder in Japan may be misleading, and almost the same incidence as that in Western countries might be expected.

1.6

Genotypes of Red Cell Membrane Disorders

The processes of advances in red cell membrane research can be well understood by reading the five special issues of *Red Cell Membrane*, which were published sequentially in the *Seminars in Hematology* from 1971 to 1993. The first issue on *Red Cell Membrane* in 1970 was edited by Weed [38], and the contents have been described previously. The second issue was published in 1979 edited by Jacob [50]. The major topic was “Membrane Proteins and Their Functions”. The third issue came out in 1983, edited by Palek [53] and considered projects on “Cytoskeletal Proteins”, when biochemical investigations appeared to have reached a level of completion. The fourth issue in 1990 dealt with topics on “Genetics in Hematology”, and was edited by Ranney [67]. In this issue, there was an excellent review on red cell membranes by Palek and Lambert [68], through which one can appreciate the increase in knowledge on membrane protein-related genes. Just after this issue, the fifth one, edited by Palek [69], was published sequentially in 1992 and 1993. There were many excellent reviews covering all fields of normal and abnormal red cell membranes in this program (Table 1.2). Progress on the same topics can be traced back through the issues of this journal.

Regarding the α -spectrin gene (SPTA1), the normal structure and functions of this gene including its promoter region, were clarified by Forget et al., Sahr et al. (1990), Kotula et al. (1991) and others [70–74]. The mutations of SPTA1 were chiefly detected in HE patients, and were mostly missense mutations in heterozygous states. In HPP patients, on the other hand, they were observed in homozygous states. The SPTA1 mutations (Fig. 1.5) were observed chiefly at the NH₂-terminal region corresponding to the protein abnormalities. The low expression gene (α^{LELY}) of SPTA1 is of critical importance in gene expression in HE patients.

Table 1.2 Genetic characteristics of membrane protein-related genes in human erythroid cells.

<i>Protein</i>	<i>Gene symbol</i>	<i>Chromosome location</i>	<i>Amino acids</i>	<i>Gene (kb)</i>	<i>Exons</i>	<i>Related diseases</i>
α -Spectrin	SPTA1	1q22–q23	2429	80	52	HE, HPP, HS
β -Spectrin	SPTB	14q23–q24.2	2137	>100	32	HE, HPP, HS
Ankyrin	ANK1	8p11.2	1881	>120	42	HS
α -Adducin	ADDA	4p16.3	737	85	16	
β -Adducin	ADDB	2p13–p14	726	~100	17	
Band 3 (AE1)	EPB3 (SLC 4A1)	17q21–q22	911	17	20	HS, SAO, RTA
Protein 4.1	EL1 (EPB41)	1p36.1	588	>250	23	HE
Protein 4.2	ELB42	15q15	691	20	13	HS variant
Dematin	EPB49	8p21.1	383	—	—	
p55	MPP1	Xq28	466	—	—	
β -Actin	ACTB	7p12–p22	375	>4	6	
Tropomodulin	TMOD	9q22.2–q22.3	359	—	—	
G3PD	GAPD	12p13.1–p13.31	335	5	9	
Stomatin	EPB72	9q34.1	288	12	7	None
Tropomyosin	TPM3	1q31	239	—	—	
Protein 8	PRDX2	—	—	—	—	—
Glycophorin A	GYPA	4q28.2–q31.1	131	>40	7	None
Glycophorin C	GYPC	2q14–q21	128	14	4	HE
Glycophorin B	GYPB	4q28.2–q31.1	72	>30	5	None
Glycophorin D	GYPD	2q14–q21	107	14	4	HE
Glycophorin E	GYPE	4q28.2–q31.1	59	>30	4	

HE: hereditary elliptocytosis, HPP: hereditary pyropoikilocytosis, HS: hereditary spherocytosis, SAO: Southeast Asian ovalocytosis, RTA: renal tubular acidosis.

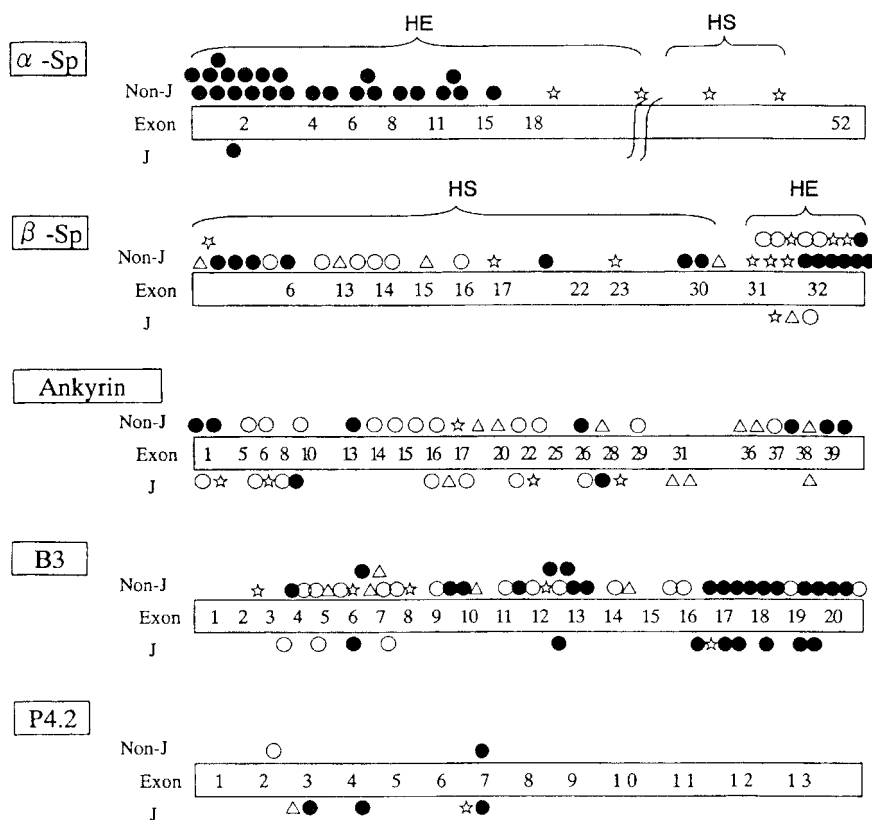


Figure 1.5 Characteristics of gene mutations of Japanese. Open circles denote frameshift mutations, closed circles missense mutations, open triangles nonsense mutations, and open star symbols abnormal splicing, respectively. HS: hereditary spherocytosis, HE: hereditary elliptocytosis, Non-J: non-Japanese, and J: Japanese.

The functional significance is discussed in detail in the text. No mutations of SPTA1 have been reported at the C-terminal side. Two mutations of SPTA1, one of which is a low expression gene (α^{LEPRA}), are known in HE.

The normal structure and functions of the β -spectrin gene (SPTB) were extensively investigated by Forget et al., which included Winkelmann (1990). More than 20 mutations are known, most of which have been detected at the C-terminal region (mostly

at exon 30 or intron 30) corresponding to the β -spectrin anomalies (Fig. 1.5). The clinical phenotype is mostly HE. To date, seven missense mutations, one nonsense mutation, six splicing abnormalities, and four frameshift mutations have been reported in these HE patients, 13 being of autosomal dominant inheritance, three of autosomal recessive inheritance, and two being *de novo* mutations [72–75].

Mutations of SPTB were also detected in HS patients by Hassoun and others: seven missense mutations, two nonsense mutations, two splicing abnormalities, six frameshift mutations, and one genomic deletion. Three SPTB mutations have been found in Japan.

The structure and functions of the ankyrin gene (ANK1) were mostly elucidated by Lux et al. (1990), and Lambert et al. (1990). Eber et al. first clarified ANK1 mutations, mostly in German HS patients. To date, more than 60 mutations have been observed including 20 mutations in our Japanese HS patients (Fig. 1.5). Most of these mutations are frameshift and nonsense mutations. The mutations are widely spread over all regions of ANK1 without so-called hot spots [72–75].

Analyses of the band 3 gene (EPB3) have largely been carried out by Tanner et al. in Bristol (1991). Mutations of this gene have mostly been observed in HS patients except for Southeast Asian ovalocytosis, the phenotype of which is common HE. To date, more than 57 mutations of EPB3 have been detected by Palek et al., Lux et al., and Delaunay et al. in France and others, including 14 mutations reported by us, which have been distributed widely on this gene (Fig. 1.5). However, missense mutations tend to be clustered at the C-terminal region [72–75].

The protein 4.1 gene (EL1) is known by its extremely complex alternative splicings, which have been studied mostly by Conboy in Berkeley. Total deficiency of protein 4.1 has been reported in four HE patients, in whom the mutations at the downstream AUG of this gene are known to be pathognomonic. Qualitative abnormalities in the protein 4.1 gene have also been reported in two categories: (1) a truncated form (protein 4.1^{68/65}), and (2) an elongated form (protein 4.1⁹⁵). In the former, the 10 kDa spectrin–actin domain is missing due to a deletion of Lys407–Gly486, and in the latter, the 10 kDa spectrin–actin domain is essentially duplicated due to an insertion of Lys407–Gln529.

The protein 4.2 mutations are almost all limited to the Japanese population, except for protein 4.2 Tozeur and protein 4.2 Lisboa which were reported by Hayette et al. in Lyon. To date, four missense mutations, one frameshift mutation, one nonsense mutation, and one donor site mutation have been detected (Fig. 1.5). The patients are homozygotes or compound heterozygotes by two different missense mutations. Most of the mutations of the protein 4.2 gene (EPB42) are of the Nippon type. The structure and functions of EPB42 were investigated extensively by Korsgren et al. in Boston, Sung et al. in San Francisco, and Takaoka and our group in Japan. A protein 4.2 variant (protein 4.2 doublet Nagano) was produced by post-translational modification in addition to the presence of a missense mutation (CGT488CAT). The expression of EPB42 and protein 4.2 during erythroid development and differentiation has been studied. mRNA was expressed in early erythroblasts, but protein 4.2 *per se* appeared in late erythroblasts (probably at the stage of orthochromatic normoblasts close to reticulocytes).

1.7

Reevaluation of Molecular Electron Microscopy for Phenotypes

Although cytosolic proteins (hemoglobin, glycolytic enzymes, etc.) demonstrate their biological activities in the cytoplasm, membrane proteins have to be assembled into the stereotactic ultrastructure of cell membranes *in situ* to demonstrate their biological functions, such as membrane transport, cell shape, and cell deformability. Therefore, in a diseased state, even though some determined gene mutations have been identified, their significance must be examined as to whether or not they can explain the determined phenotypes. Biochemical examinations may not be enough to verify the causal relationship between the genotype and the phenotype in diseased states. From this standpoint, molecular electron microscopy plays a crucial role in proving abnormalities in membrane ultrastructure *in situ*, which can be expected from the data obtained by gene analyses.

Electron microscopic examinations of the integral proteins of red cell membranes were initiated by Meryman and Kafig in 1955 utilizing the freeze-fracture method [76]. Thereafter, this method was improved by Bullivant and Weinstein, who applied it to the red cells of paroxysmal nocturnal hemoglobinuria in 1967. They found multiple small disseminated particles on the outer surface of red cell membranes and designated these particles as MAPs (membrane-associated particles). Weinstein (1970) reported that the number of MAPs was $2600 \pm 520 \mu\text{m}^{-2}$, which is fairly close to the value which is now widely accepted, approximately $5000 \pm 500 \mu\text{m}^{-2}$. MAPs are equivalent to intramembrane particles (IMPs), 80 % of which are composed of band 3 molecules. In the early 1970s, when electron microscopy with the freeze-fracture method became widely utilized, MAPs, that is IMPs, appeared to be considered erroneously as glycophorins, which were being investigated extensively at that time [77].

In normal subjects, IMPs (mostly band 3 molecules) are distributed regularly like icebergs floating on the sea with wide open channels of water between them (Fig. 1.6). The basic molecular size of approximately 80 % of IMPs is around 8 nm in diameter. This unique distribution pattern is dependent on the physiolog-



Figure 1.6 Intramembrane particles (IMPs) in normal human red cells examined by electron microscopy with the freeze fracture method.

ical restriction of band 3 molecules, which are bound to the cytoskeletal network via ankyrin (Cohen et al., 1978). In the diseased state, IMPs may be polymerized to form larger aggregates. Thus, detailed examination of the state of IMPs can clearly lead to detection of abnormalities of band 3 molecules. In mutated conditions, these observations were made with a total deficiency of band 3 (Inaba, et al., 1996 [78], Peters et al., 1996, and Palek et al., 1996), with a total deficiency of protein 4.2 (Yawata, 1994 [79]), and in the rare homozygous missense mutation of band 3 Fukuoka (Inoue et al., 1998) [80].

Not long after electron microscopy with the freeze-fracture method had been established, detection of the cytoskeleton in red cell membranes was attempted in parallel. Cohen and Branton et al. (1975), and Bennett et al. (1977) reported the presence of the cytoskeleton electron microscopically [55]. In 1976, Lux et al. reported the detection of fibrous spectrins in the red cell membrane structure utilizing electron microscopy [81]. Their findings were followed by those of Sheetz and Singer (1977), Ralston et al. (1978), and Shotton (1979). The presence of the cytoskeletal network was verified by Tsukita et al. (1980) using electron microscopy with the thin-section method, and by Nermut (1981), and Byers and Branton (1985) using electron microscopy with the negative staining method [82].

During this same period, red cell membrane proteins were first extracted and purified, and structural analyses of these red cell membrane proteins began, initially with spectrin molecules [51, 55, 82, 83]. By utilizing electron microscopy with the negative staining method, a monomer, a dimer, and a tetramer of the extracted spectrins were analyzed by many investigators, including Branton (1978), Byers (1985), Shen (1986), and Liu (1987). Biochemical results on the formation of heterotetramers ($\alpha_2\beta_2$) from heterodimers ($\alpha\beta$) of the extracted α - and β -spectrins were verified and visualized by electron microscopy. At this point, studies on membrane proteins expanded from ones on a single molecule to ones on the interactions of these membrane proteins *in situ*.

It was not long until the cytoskeletal network was discovered with a basic unit of hexagonal forms. Immuno-electron microscopy with antibodies against various purified membrane proteins made great contributions to identification of the exact localization of these membrane protein *in situ*, as published by Liu et al. (1987), and Derick et al. (1992). Electron microscopy with the negative staining method was an excellent tool for comprehending the structures and functions of these extracted, purified membrane proteins *in vitro*.

With clarification of the overall view of the cytoskeletal network in normal red cell membranes, studies on the red cell membranes in diseased states were started, especially by Liu, who treated red cell ghosts of hereditary elliptocytosis and of hereditary pyropoikilocytosis at a low ionic strength or a low concentration of Triton X-100, and examined them by electron microscopy with the negative staining method. He first observed disruption of the cytoskeletal network in these patients (Liu et al., 1990 and 1992 [84]).

As mentioned previously, although this negative staining method was an excellent tool for studies on extracted membrane proteins, there was a theoretical limitation to its methodology in that the cytoskeletal network had to be artificially ex-

tended by various detergents. Therefore, electron micrographs obtained by this method may not exhibit the true native unimpaired state of the cytoskeletal network *in vivo*. For this purpose, electron microscopy with the quick-freeze deep-etching (QFDE) method was developed, and applied to the examination of the cytoskeletal network in normal red cell membranes *in situ* (Fig. 1.7) by Pumpplin et al. (1990) and Ursitti et al. (1991) [85]. With this method, electron microscopic images of red cell membranes became extremely clear and sharp, and could be easily examined qualitatively and quantitatively.

The first application of this method to diseased states was made by our group. For better qualitative and quantitative evaluations, limited disorders were selected, such as cases of genetically identified homozygous mutations with total deficiencies of the determined membrane proteins. In this category, total deficiency of protein 4.1 (protein 4.1 Madrid: Yawata et al., 1997 [86]) was evaluated with regard to the cytoskeletal network, total deficiency of bovine band 3 (Inaba et al., 1996 [78]) was examined with regard to the integral proteins, and the total deficiency of protein 4.2 (Inoue et al., 1994, and Yawata et al., 1996 [87]) was evaluated. Studies were further extended to homozygous band 3 Fukuoka (Inoue et al., 1998 [80]), and β -spectrin Yamagata (β -Sp^{220/214}) in hereditary elliptocytosis (Yawata et al., 1994 [88]).

For immuno-electron microscopy *in situ*, we developed a technique of electron microscopy using the surface-replica method (Yawata et al., 1994) [88]. This procedure is carried out at room temperature, which means that the method is applicable to immuno-electron microscopy. The quality of images is fairly good. Using this method, we employed immuno-electron microscopy to study Ankrin Marburg and Ankyrin Stuttgart (Yawata et al., 1999). This method can verify the functional significance of determined genotypes in the red cell membrane structure *in situ*.

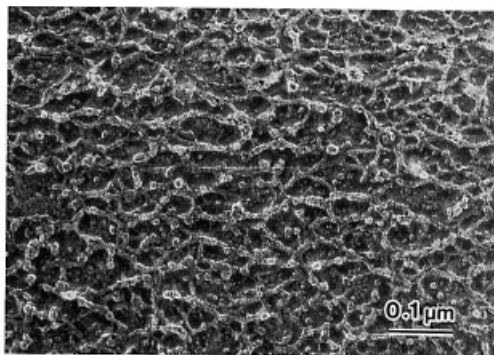


Figure 1.7 Cytoskeletal network of normal human red cells examined by electron microscopy with the quick-freeze deep-etching method.

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2

Composition of Normal Red Cell Membranes

2.1

Introduction

Some of the components of the red cells exist in entirety in the structural portion of the cell, although it would appear that others occur in both the membrane and the free fluid of the cell. Therefore, the method of separation of the cell membrane definitely determines whether or not a cell component appears as part of the structure or free in the cellular fluid. This clearly indicates that some cell components may be associated with, but may not be an essential part of, the membrane ghost.

The substantial quantities of proteins, lipids, and carbohydrates, which are the components of red cell membranes, are closely associated with one another in the membranes.

Fractions of red cell ghosts containing various proportions of proteins, lipids, and carbohydrates have been prepared by many techniques. None have been proven to be discrete entities. However, for membrane protein analyses, red cell ghosts can be prepared basically by the hypotonic lysis method, and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) utilizing the methods of Fairbanks et al. [1], and of Laemmli [2]. Even with this procedure, preparation of membrane proteins from red cell ghosts was difficult due to the ease with which the peripheral proteins can be lost when red cell membrane ghosts were washed.

Dodge et al. [3] demonstrated that all the lipids in red cells could be recovered in completely hemoglobin-free membrane ghosts prepared by lysis and washing with a hypotonic phosphate buffer. It is now generally accepted that, in red cells in nature, all the cellular lipids reside in the cell membrane.

It has been reported that the red cells are composed of water ($721 \pm 17 \text{ mg mL}^{-1}$ of the red cells), proteins (371 mg mL^{-1} of the red cells), most of which is hemoglobin (335 mg mL^{-1} of the red cells), lipids (5.1 mg mL^{-1} of the red cells), and carbohydrates (80 mg mL^{-1} of the red cells). The red cell membranes are composed of 19.5 % (w/w) water, 39.6 % proteins, 35.1 % lipids, and 5.8 % carbohydrates, the relative contents of which appear to be similar to those in most animal membranes [4].

Disregarding the water content, which remains inside the membrane ghosts, intact red cell ghosts contain 49.2 % (w/w) protein, 43.6 % total lipids (32.5 % phos-

pholipids and 11.1% cholesterol), and 7.2% total carbohydrates (4.0% neutral sugars, 2.0% hexosamines, and 1.2% sialic acids) [4].

2.2

Membrane Lipids

Composition and functions of membrane lipids are described in this Section, but their biophysical characteristics are also described in the Introduction of Chapter 17 (Section 17.1) in normal and disease states.

2.2.1

The Contents and Nature of Membrane Lipids

The content of total lipids is approximately 5.0×10^{-10} mg per red cell, in which phospholipids are about 60%, neutral lipids (chiefly free cholesterol) 30%, and the rest is glycolipids. The molar ratio of cholesterol:phospholipids is 0.90 (Table 2.1).

In human red cells, phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and phosphatidylserine (PS) are predominant, that is, approximately 31% (w/w), 29%, 26%, 13%, respectively [5–8]. Minor components are phosphatidylinositol (PI), PI-monophosphate (PIP), PI-4,5-bisphosphate (PIP₂), phosphatidic acid (PA), lysophosphatidylcholine (lyso-PC), lysophosphatidylethanolamine (lyso-PE). At the physiologic pH, PS, PA, and PI have a net negative charge, although the other major phospholipids are electrically neutral.

Table 2.1 The contents of plasma lipids and red cell membrane lipids in normal human beings.

(A) Plasma lipids

Cholesterol (mg dL ⁻¹)	
Total	178 ± 32
Free	49 ± 9 (27.5 ± 5.0%)
Esterified	129 ± 23 (72.5 ± 12.9%)
High-density-lipoprotein	
Cholesterol (mg dL ⁻¹)	50 ± 11
Phospholipids (%)	
Lysophosphatidylcholine	4.8 ± 2.4
Phosphatidylcholine	69.2 ± 6.3
Sphingomyelin	17.2 ± 2.7
Phosphatidylethanolamine	3.4 ± 3.1
Phosphatidylserine + phosphatidylinositol	2.3 ± 2.3
Others	2.2 ± 2.6
Triacylglycerols (mg dL ⁻¹)	95 ± 65
Lipoprotein X	—

Table 2.1 Continued.

(B) Red cell membrane lipids	
Free cholesterol (FC)	1202 ± 103
Total phospholipids (PL)	2604 ± 241
Lysophosphatidylcholine (L – PC)	34 ± 18 (1.3 ± 0.7 %)
Phosphatidylcholine (PC)	747 ± 73 (28.7 ± 2.8 %)
Sphingomyelin (SM)	674 ± 49 (25.9 ± 1.9 %)
Phosphatidylethanolamine (PE)	805 ± 42 (30.9 ± 1.6 %)
Phosphatidylserine (PS) + phosphatidylinositol (PI)	344 ± 34 (13.2 ± 1.3 %)
PC + SM + L-PC/PE + PS + PI	1.27 ± 0.04
FC/PL ratio	0.90 ± 0.04
SM/PC ratio	0.90 ± 0.07

The numbers represent μg per 10^{10} red cells.

The numbers in parentheses represent the percentage of phospholipids (mean value $\pm$ 1 SD).

Except for SM and lyso-PC, most phospholipids have two fatty acid chains, which are attached to a glycerol backbone. The state of saturation (saturated or unsaturated) of fatty acids, and the length of these acyl chains should affect the degree of membrane fluidity significantly, as described in the following chapters. The lyso-phospholipids have only one fatty acid, and demonstrate strong hemolytic properties, especially on lyso-PC.

Contrary to these results in human red cells, it is interesting to note that PC is totally absent in the red cells of sheep and is reduced markedly in camels, and in the red cells of rats it is significantly increased (47.5 %) [6, 9]. The relative contents of PE (around 25 %) and PS (approximately 15 %) are fairly consistent in the red cells of these animals. The exact reason why these discrepancies exist in the various species of mammals and its biological significance in their membrane functions have not been elucidated.

Comparative fatty acid patterns of total lipids of red cells from various animal species [6, 9] demonstrate that in human red cells, the fatty acid (16:0) is predominant, but also other fatty acids (18:0, 18:1, 18:2, and 20:4) are present at almost the same levels [7, 8] (Table 2.2). However, in the red cells of sheep, the fatty acid (18:1) is significantly increased, but other fatty acids (16:0, 18:0, 18:2), and particularly the fatty acid (20:4), are at a minimum. In contrast, in rat's red cells, the predominant fatty acids are 16:0 and 20:4 [6]. These striking differences in lipid composition may have important implications for the membrane properties of the different cells.

It is known that phospholipids such as amphipathic lipids contain aliphatic carbon chains or tails at one end of the molecule which attach to polar head groups with more highly charged moieties [6, 9]. The hydrophobic tails of these molecules exclude water by associating together and forming a nonaqueous interior. The polar head groups keep contact with the aqueous solution however. This molecular mechanism leads to the formation of micelles. Membrane lipids are generally composed of highly polar head groups and enormous hydrophobic tails, which allow

Table 2.2 Fatty acid composition of plasma lipids and red cell membrane lipids in normal human beings.**(A) Fatty acids of plasma lipids**

	<i>Total lipids</i>	<i>Phosphatidylcholine (PC)</i>	<i>Sphingomyelin (SM)</i>	<i>Phosphatidylethanolamine (PE)</i>
DMA	—	—	—	5.7 ± 1.1
16:0	21.0 ± 1.1	28.9 ± 1.1	25.4 ± 0.4	14.4 ± 1.4
16:1	1.6 ± 0.9	—	—	—
DMA	—	—	—	5.7 ± 0.7
18:0	7.5 ± 0.4	14.5 ± 0.3	7.7 ± 0.7	13.9 ± 1.2
18:1	18.6 ± 2.3	10.5 ± 0.5	4.3 ± 1.3	10.2 ± 1.4
18:2	33.5 ± 4.5	26.1 ± 2.4	6.3 ± 2.3	19.0 ± 3.2
20:0	—	—	4.1 ± 0.6	—
20:1	—	—	—	—
20:3	1.0 ± 0.3	2.2 ± 0.5	—	—
20:4	5.7 ± 0.6	7.1 ± 0.7	—	11.2 ± 1.7
20:5	1.7 ± 0.6	2.4 ± 0.6	—	2.4 ± 0.7
22:0	—	—	11.3 ± 2.2	—
22:1	—	—	1.7 ± 0.5	—
22:2	—	—	4.7 ± 0.8	—
22:5	—	—	1.9 ± 0.4	1.1 ± 0.3
22:6	4.0 ± 0.7	5.3 ± 0.5	—	9.7 ± 1.7
24:0	0.5 ± 0.1	—	7.9 ± 1.0	—
24:1	0.9 ± 0.1	—	19.9 ± 2.6	—

(B) Fatty acids of red cell membrane lipids

	<i>Total lipids</i>	<i>Phosphatidylcholine (PC)</i>	<i>Sphingomyelin (SM)</i>	<i>Phosphatidylethanolamine(PE)</i>	<i>Phosphatidylserine + phosphatidylinositol</i>
DMA	—	—	—	4.8 ± 0.3	—
16:0	21.8 ± 1.2	34.9 ± 0.8	26.2 ± 1.1	16.0 ± 0.5	3.8 ± 0.7
DMA	—	—	—	8.0 ± 0.9	—
18:0	14.3 ± 0.4	12.2 ± 0.4	7.1 ± 0.5	8.7 ± 0.2	46.7 ± 2.8
18:1	13.4 ± 1.0	17.2 ± 0.6	2.4 ± 0.6	17.0 ± 0.8	8.5 ± 0.7
18:2	10.8 ± 1.0	22.6 ± 1.0	2.2 ± 0.8	6.6 ± 1.2	2.3 ± 0.2
20:4	12.0 ± 0.8	4.6 ± 0.6	—	16.4 ± 0.9	18.2 ± 0.7
20:5	1.5 ± 0.5	1.6 ± 0.4	—	3.0 ± 0.7	0.9 ± 0.4
22:0	—	—	7.0 ± 0.4	—	—
22:4	—	—	—	3.4 ± 0.5	2.0 ± 0.3
22:5	2.8 ± 0.3	—	4.4 ± 0.5	3.8 ± 0.4	3.2 ± 0.4
22:6	7.2 ± 0.9	2.9 ± 0.4	—	9.5 ± 0.9	10.8 ± 0.9
24:0	4.9 ± 0.7	—	19.1 ± 0.8	—	—
24:1	4.4 ± 0.9	—	26.6 ± 1.5	—	—

Values are percentages. DMA : dimethylacetal derivatives.

them to exist in the hydrophobic environment of a membrane lipid bilayer. The most amphipathic phospholipids are phosphoglycerides and SM.

The glycolipids in human red cells are mostly based on sphingosine, such as glycosphingolipids [6, 9]. Typical arrangements of sugar residues on the lipid core result in the formation of gangliosides. Glycolipids are located almost exclusively on the extracellular face of the lipid bilayer, and the sugar residues stick out into the extracellular space. They carry several important red cell antigens, such as A, B, H, Le^a, Le^b, and P and may also provide other important functions [10]. Glycolipids and cholesterol are intercalated between the phospholipids in the bilayer, with their long axes being perpendicular to the lipid bilayer.

2.2.2

Asymmetry of the Membrane Lipid Bilayer

In human red cells, PS ($96 \pm 4\%$) and PE ($80 \pm 5\%$) are almost exclusively located in the inner monolayer of the lipid bilayer, while PC ($30 \pm 7\%$) and SM (approximately 10%) are present minimally in the inner monolayer. Therefore, PC and SM are predominantly located in the outer monolayer [5, 6].

This lipid asymmetry is a widespread property of eukaryotic membranes, and appears to play a critical role in the normal interaction of the cell with its outer environment [11, 12]. The best example is the appearance of PS on the outer surface which may lead to a thrombotic diathesis through its binding to prothrombinase, or to apoptosis with the complement activation. The complement induces apoptosis of the cell by the appearance of PS on the outer surface.

This asymmetry appears to depend on the activity of flippase [13], which actively translocates PS and PE to the inner leaflet, and that of floppase [14, 15], which catalyzes translocation to the outer leaflet of the bilayer. Flippase is a member of the Mg²⁺-dependent, P-glycoprotein ATPases. Floppase is a multidrug resistance protein 1 (MRP1). An enzyme, scramblase, has also been isolated [16], and which is proposed as being responsible for the redistribution of membrane phospholipids under the activation of calcium via an EF motif. A defect in phospholipid scramblase has been found in Scott syndrome, in which activated platelets fail to expose phosphatidylserine on their surface sufficient for assembly of prothrombinase resulting to bleeding tendency [17].

Red cell lipids also exist in different domains within each of the bilayer planes; that is a *cis* asymmetry, related to macroscopic and microscopic domains of the membrane lipids [18–19]. These lipid-rich domains are intrinsic structural features of the membrane. Lipids also partition on a microscopic scale within the membrane. Positively charged amino acids are concentrated on the cytoplasmic side of glycophorins, because glycophorin A binds anionic (phosphatidylserine and phosphatidylinositol) but not choline (phosphatidylcholine and sphingomyelin) phospholipids. Anionic phospholipids appear to cluster near the regions of positive charge. Other surface-bound membrane proteins (spectrin and protein 4.1) also prefer to bind to anionic phospholipids. The membrane microdomains or rafts produce lateral heterogeneity in the lipid bilayer of the plasma membrane in

various signaling processes in cell membranes [20–23]. Typical examples have been demonstrated as a complex mixture of membrane proteins including stomatin, the Duffy protein receptor, glycosylated phosphatidylinositol-anchored proteins (GPI-proteins: CD55, CD58, and CD59). Cholesterol depletion from the red cell membrane abolishes this raft formation by losing the ability to produce membrane microdomains. Therefore, membrane lipid rafts may function for the membrane remodeling on its surface.

In red cell membranes of various species of mammals, the asymmetry of the distribution of these phospholipids has been observed as being a fairly common phenomenon. The extent of this asymmetry, however, is variable, that is, PC at the outer leaflet of pig's platelets is about 40 % of the total PC, and only 20 % of the total PC is present at the brushborder of a rabbit's gut [6]. The biological significance of the variation of phospholipid distribution among the various species of mammals is not known at the present time. Among these species, PS and PE are almost consistently located in the inner leaflet of the membrane lipid bilayer of various tissues [11, 12].

2.2.3

Membrane Fluidity

The long hydrocarbon tails of phospholipids can be modified to varying extents by the formation of double bonds between carbon moieties, which create unsaturated phospholipids. The state of saturation of fatty acids should affect the extent of membrane fluidity significantly [24]. As the degree of desaturation increases, the packing of hydrophobic tails in the core of the bilayer is increasingly disrupted, thereby enhancing the membrane fluidity.

Membrane lipid fluidity is basically dependent on several factors (Table 2.3), these are: the type of cholesterol (free or esterified), the class of phospholipids,

Table 2.3 Determinants of red cell membrane fluidity

	<i>Softening factors</i>		<i>Hardening factors</i>
Type of cholesterol	Esterified		Free (unesterified)
Molar ratio of cholesterol/ phospholipids	0.60	← (1.00) →	1.60
Type of phospholipids	Increased PC		Increased FC and SM
Saturation of fatty acids	Increased unsaturation		Increased saturation
Length of acyl chains	Increased shorter chains (C : 14~16)		Increased longer chains (C : 20~24)
Content of Lyso-PC	Increased Lyso-PC		Decreased Lyso-PC
Presence or absence of amphipathic compounds	Presence		Absence

FC: free (unesterified) cholesterol, PC: phosphatidylcholine, SM: sphingomyelin,
Lyso-PC: lyso-phosphatidylcholine.

the molar ratio of cholesterol to phospholipids, the degree of saturation of fatty acids, as discussed above, the length of the acyl chains, and the presence or absence of amphipathic compounds such as lysophosphatides [24]. Free cholesterol (FC) decreases the membrane fluidity, while esterified cholesterol (EC) increases it. PC increases the membrane fluidity, and SM and PE decrease it. Short acyl chains and low saturation of fatty acids increase membrane fluidity, and longer acyl chains and high saturation of fatty acids decrease it. Lysophosphatides increase membrane fluidity. Compensation for an alteration to one or more of these variables to maintain normal membrane fluidity has been termed “homeoviscous adaptation” by Sinensky [25]. This phenomenon has been observed in *Escherichia coli*, fungi (*Fusarium*), *Tetrahymena*, hibernating squirrels, guinea pig lymphatic leukemia cells, and human red cell membrane disorders such as congenital lecithin:cholesterol acyltransferase (LCAT) deficiency [7] and hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCA) [8], as described in detail in the Sections 17.2 and 17.4 of this monograph.

2.2.4

Renewal of Membrane Lipids

Mature human red cells cannot synthesize membrane lipids (fatty acids, phospholipids, or cholesterol) *de novo*. Therefore, they depend on lipid exchange and acylation of fatty acids as the mechanisms for phospholipid repair and renewal [5, 6]

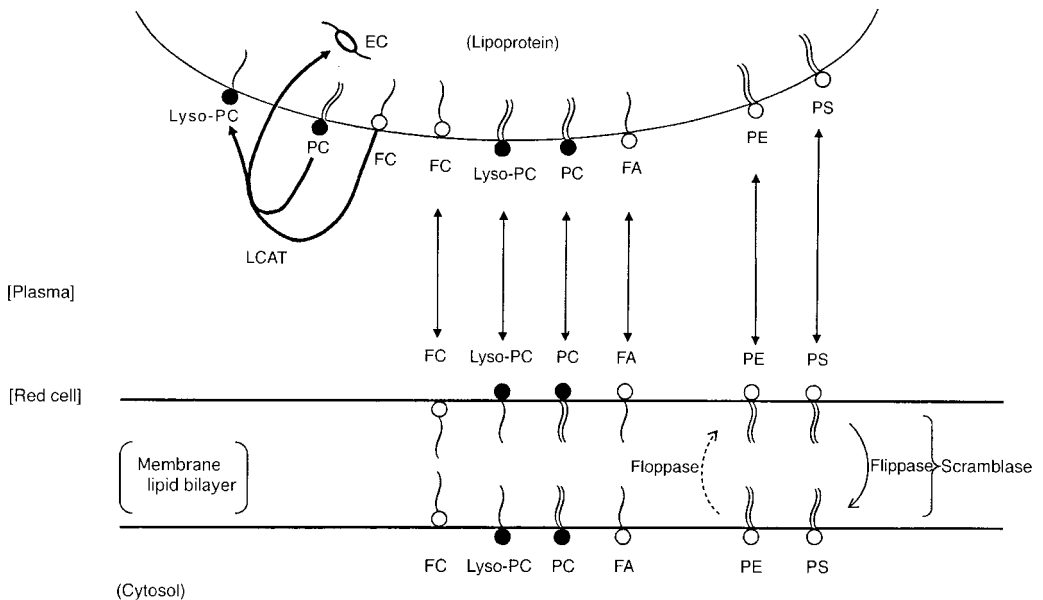


Figure 2.1 Schematic pathways for lipid exchange between plasma and red cell membranes. PC: phosphatidylcholine, FC: free cholesterol, EC: esterified cholesterol, Lyso-PC:

lyso-phosphatidylcholine, FA: fatty acids, PE: phosphatidylethanolamine, PS: phosphatidylserine, and LCAT: lecithin:cholesterol acyltransferase.

(Fig. 2.1). The exchange rate of outer bilayer phospholipids (PC and SM) with the phospholipids of lipoproteins in plasma is extremely slow (a turnover time of 5 days) [26]. Phospholipids (PS and PE) at the inner leaflet of the bilayer are essentially nonexchangeable.

Free cholesterol (FC) in red cell membranes exchanges readily with the unesterified FC in plasma lipoproteins (a half-life of 7 h) as reviewed in [6]. FC is also in part converted into esterified cholesterol (EC) by the enzymatic process of lecithin:cholesterol acyltransferase (LCAT). The EC, which is produced *de novo* by the reaction, is transferred out to the plasma. Therefore, LCAT catalyzes a unidirectional pathway that depletes the membrane cholesterol and decreases its surface area. When LCAT is absent, as in LCAT deficiency discussed in Section 17.2, excessive membrane cholesterol accumulates, and expands the membrane surface area, producing stomatocytosis [7].

The fatty acid acylation pathway is an energy (ATP)-dependent process. By this pathway, fatty acids are incorporated into lysophosphatides (mostly lyso-PC) to produce the natural phospholipid with two acyl chains. The enzyme (acylase) and the products (phospholipids) are present in the inner leaflet of the bilayer. Through these pathways, membrane lipid components are slowly replaced. It is known that approximately 30 days are required before red cell lipids reach equilibrium after a change in dietary fatty acids [6].

2.2.5

Interactions Between Membrane Lipids and Proteins

Membrane lipids can interact with integral membrane proteins within the lipid bilayer [27]. The transmembrane segments of these integral membrane proteins (especially band 3 protein) maintain multi-component channels and membrane transport pumps through stable associations with one another within the lipid bilayer. Other proteins, particularly protein 4.1, also interact with lipids such as phosphatidylinositol 4,5-bisphosphate (PIP₂) forming the so-called “PI-turnover” [28].

Some membrane proteins, such as glycophorin A (GP-A) bind anionic (PS, PI) but not choline (PC, SM) phospholipids. Positively charged amino acids are predominantly concentrated on the cytoplasmic side of the bilayer-spanning domains of glycophorins and other membrane proteins. Anionic phospholipids (PS, PI) cluster near these regions of positive charge. Spectrin and protein 4.1, which are surface-bound, bind preferentially to anionic PS and PI, resulting in a nonrandom topography at the inner leaflet of the bilayer. The exact mechanism for this nonrandom topography of phospholipids and proteins, however, should be elucidated in detail in the near future.

2.2.6

Membrane Lipids as a Determinant of Red Cell Shape

The red cell shape is also dependent on the state of the membrane lipids. A minimal change (less than 0.4 %) in the surface area of the inner and outer leaflets can affect the shape of the lipid bilayer. Based on these facts, the lipid bilayer couple

hypothesis was proposed by Sheetz and Singer [29]. The phenomenon reflects the tight packing of membrane lipids, the independent motion of lipids in the inner or outer half of the bilayer, and the extreme thinness of the membrane (approximately 8 nm in thickness). Membrane spiculation can be produced by processes that either expand the outer bilayer or that contract the inner leaflet, resulting in spiculated red cells (echinocytes) by exocytosis (Fig. 2.4). However, when the inner leaflet is subjected to a relative expansion, membrane invagination (endocytosis) should occur by producing cup-shaped red cells (stomatocytes) [30] (Fig. 2.4). Strongly charged amphipathic compounds such as phospholipids cause echinocytosis, by being trapped into the outer leaflet of the lipid bilayer by their fixed charge. Some amphipathic compounds, which are permeable and can cross the membrane in their uncharged form, cause the membrane to extend toward the side of greater accumulation [31]. It is a general rule that cationic compounds accumulate in the inner leaflet, which is negatively charged, and anionic compounds are distributed in the outer leaflet which is neutral. This variation is extremely sensitive, and can produce a change in the shape of the red cells. There have been numerous reports on the *in vitro* experiments utilizing various amphipathic compounds (chlorpromazine, vinblastine, etc.), echinocytogenic agents (barbiturates, salicylates, saponin, ethanol, calcium, etc.), or stomatocytogenic agents (lecithin, cholic acid, primaquine, colchicine, Triton-X, etc.) [32]. Although this coupled bilayer hypothesis is very attractive, and can explain a number of phenomena well, a serious question has recently been raised by Nakao [33]. The facts are as follows: human red cells are stomatocytic at pH 5.5, diskoid at pH 7.0, and crenated at pH 7.9. The shape of ghosts obtained by osmotic hemolysis is quite different, that is, crenated at pH 5.5, diskoid at pH 7.0, and stomatocytic at pH 7.9. The discrepancy in the results cannot be explained adequately by the coupled bilayer hypothesis alone [33]. Gedde et al. [34] have recently demonstrated that the resealed ghosts with normal ATP level behaved much like intact red cells, not like unincubated ghosts. This may indicate that the shape of red cells is primarily determined by membrane proteins, especially the spectrin network, but also secondarily by the lipid bilayer.

2.3

Membrane Proteins

2.3.1

Separation and Identification of Membrane Proteins

The red cell membranes contain more than ten major proteins, and probably hundreds of minor proteins. These proteins are usually extracted from red cell ghosts, which are washed three times with the isotonic saline and are subjected to hypotonic hemolysis with mild detergent to remove the membrane lipids [3]. The red cell ghost proteins are separated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) [1, 2] (Fig. 1.3). The individual protein fractions are

named according to their electrophoretic mobility by the SDS–PAGE, as described by Fairbanks [1]. The top (slowest) migrating band is band 1, the next slowest band is band 2, and so on. Sub-bands are designated with decimals, that is, protein 4.1 and protein 4.2, which are two sub-bands constituting a region at the position of the fourth migrating band. The protein bands are named logically from 1 to 7. Since protein chemistry has characterized the nature of these proteins, these proteins have thus been renamed to indicate their biochemical nature and functions more precisely (Table 1.1). For example, band 1 and 2 are the α - and β -chain of spectrin, band 2.1 is ankyrin, band 3 is the anion transport exchanger (AE-1), and protein 5 is actin. Some of major membrane proteins (proteins 4.1 and 4.2) are recognized by utilizing their numerals as they stand. Although it has been known that numerous membrane proteins are identified as protein bands on the SDS–PAGE gels, the most notable membrane proteins are the two spectrin sub-units (α - and β -chain), ankyrin, the adducins, band 3, protein 4.1, protein 4.2, protein p55, actin, protein 7.2b (stomatins), and the glycophorins. These membrane proteins are easily identified on the SDS–PAGE gel by the staining method using Coomassie-blue, except for the glycophorins, which can only be detected by the staining method using Periodic acid Schiff (PAS) (Fig. 1.3).

2.3.2

Membrane Proteins and Membrane Structure

Membrane proteins are categorized into two groups according to the ease with which they can be removed from the membranes [35]. Some of them are more loosely associated, and they are easily removed by high- or low-salt or high-pH extraction, probably because they appear to be associated with only one face (exterior or interior) of the membrane. This group of membrane proteins is called the “peripheral proteins”. The second group of membrane proteins, “integral proteins”, can be extracted only by harsh reagents (chaotropic solvents or detergents), probably because they are tightly embedded into or through the lipid bilayer by hydrophobic domains within their amino acid sequences. The representative peripheral proteins are spectrins (α - and β -chains), and the most typical examples of integral proteins are band 3 and glycophorins (Fig. 2.2).

Membrane proteins are also classified into three categories according to their functional properties in the membrane ultrastructure. The first ones are cytoskeletal proteins. Typical examples are spectrins (α - and β -chains), protein 4.1, and actin. They are specifically associated with one another to form a cytoskeletal network, which is localized just beneath the lipid bilayer, as visualized by electron microscopy (see Section 3.2.2). The second are integral proteins. The representative proteins are band 3 and glycophorins, which are firmly embedded into the lipid bilayer. The third are anchoring proteins, that is, ankyrin and protein 4.2. These membrane proteins connect with the cytoskeletal network and the integral proteins.

Considering the arrangement of the major membrane and cytoskeletal proteins within and attached to the red cell membranes, the NH_2 -terminus of the α -chain of

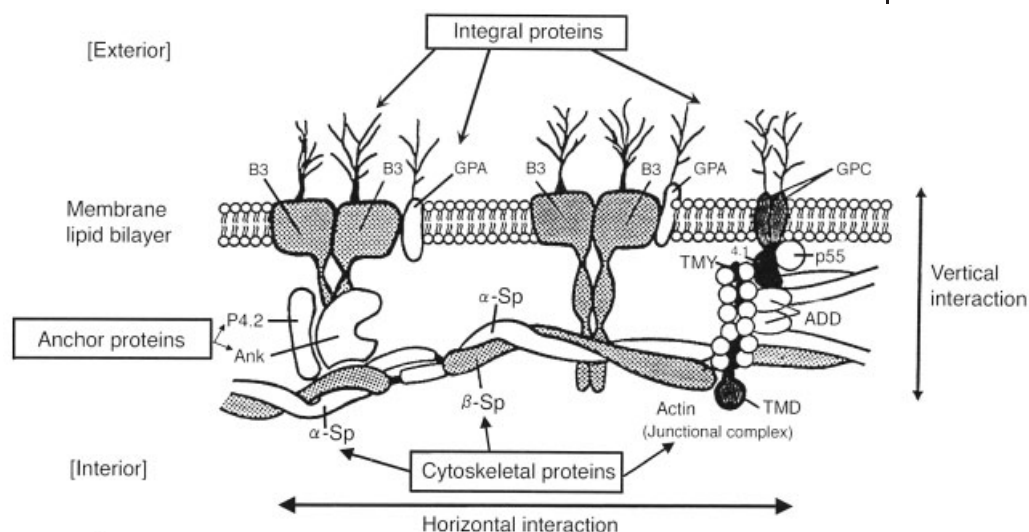


Figure 2.2 Topographical localization of human red cell membrane proteins and their interactions. Integral proteins; B3: band 3, GPA: glycoporphin A, and GPC: glycoporphin C. Anchor proteins; Ank: ankyrin, and P4.2: protein 4.2. Cytoskeletal proteins; α-Sp: α-spectrin, β-Sp: β-spectrin, ADD: adducin, 4.1: protein 4.1, TMY: tropomyosin, and TMD: tropomodulin.

spectrin interacts with the COOH-terminus of the β-chain of spectrin, that is, a spectrin dimer–dimer interaction resulting in the formation of the tetramer of spectrin ($\alpha_2\beta_2$) [36]. Attachment of the cytoskeletal network to the membrane is mediated by the formation of spectrin/actin/protein 4.1 junctional complexes, which in turn attach to the cytoplasmic domain of glycoporphin, and the binding of ankyrin to both spectrin and the cytoplasmic domain of band 3, an interaction that is facilitated by protein 4.2 [37].

The extent of the membrane protein functions is regulated by the state of phosphorylation, methylation, glycosylation, even or lipid modification (myristylation, palmitylation, or farnesylation) in these protein molecules [38]. Expression of membrane proteins are also under the control of genetic and epigenetic (gene phosphorylation, acetylation, methylation, and others) modification of membrane protein genes.

2.3.3

Membrane Proteins in the Red Cell Surface

Sialic acid residues are abundant in the red cell surface, which is negatively charged. These residues are present mostly on glycoporphin A but also on other glycoporphins, band 3 (the anion exchanger), and glycolipids [10, 35]. Glycoporphins (glycoporphins A, B, C, and D) are four sialic acid-rich glycoproteins and are a class of integral proteins [39, 40]. They constitute approximately 2% of the total

red cell membrane protein content. The gene codings for glyophorins A and B are located on chromosome 4, and those for glyophorins C and D on chromosome 2 (Table 1.2). Glyophorins consist of three domains: a cytoplasmic domain, which contains a cluster of basic residues that are located near the plasma membrane; a hydrophobic domain, which exists as a single α -helix spanning the lipid bilayer; and an extracellular domain, which is heavily glycosylated and has residues on the cell surface. Carbohydrates which are located near or on the cell surface, impart a strong net negative charge to the cell surface. The negative charge reduces the interaction of red cells with one another, the same as with other blood cells and vascular endothelial cells. Glyophorin C interacts with protein 4.1 and p55.

Blood type antigens are also located on the red cell surface [10]. MN blood group antigens reside on the glyophorin A molecule, the Ss antigen on glyophorin B, and the Gerbich blood group antigen on glyophorin C. The Rh blood group antigens are carried by a family of nonglycosylated but palmitoylated membrane proteins consisting of Rh 30 (RhD and RhCE) polypeptides and the Rh 50 glycoprotein. Other blood type antigens (Duffy, Kell, Kidd, Lutheran, Lewis, and many others) are known to reside on the red cell surface. The details are described in Section 5.3, and also in Section 15.3 in disease states.

Glycosylphosphatidylinositol (GPI)-anchored membrane proteins are embedded in the outer leaflet of the lipid bilayer [41]. A hydrophobic GPI anchor connects externally exposed hydrophilic proteins with the hydrophobic lipid bilayer.

Numerous biologically important GPI-linked surface proteins are known, such as CD59, acetylcholinesterase, leukocyte alkaline phosphatase, the CD4 antigen, and many others. Among these, the most important proteins are a group of complement-regulatory proteins in clinical hematology. Defective biosynthesis of the GPI anchor (due to mutations of the *pig-A* gene that encodes a key intermediate in the first step of this anchoring) means that these proteins are unable to become attached to the membrane, causing increased susceptibility to intravascular hemolysis by complement. The disorder is well known as paroxysmal nocturnal hemoglobinuria (PNH) [41] (see Section 5.4).

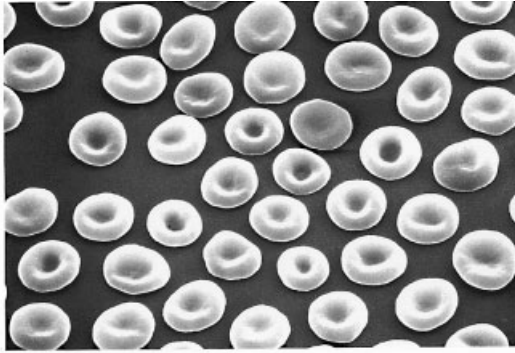
2.3.4

Membrane Proteins and Membrane Functions

2.3.4.1 Red Cell Morphology and Shape Change

Normal red cells demonstrate a unique biconcave disk shape (a discocyte) (Fig. 2.3), which allows the red cell to undergo marked deformation with a constant surface area [35]. The normal mature red cells of human adults have a mean red cell corpuscular volume (MCV) of 90 fL, a mean red cell hemoglobin concentration (MCHC) of 33 %, and a surface area of 140 μm^2 . In a pathological situation, such as spherocytosis, the surface area of these red cells would be 98 μm^2 with an MCHC of 40 %. Therefore, the diskoid shape gives a surface area of half as much again as would a spherocyte of equivalent volume. The excess surface area (approximately 40 μm^2) in normal red cells allows it to undergo marked deforma-

Figure 2.3 Normal discocytes by scanning electron microscopy.



tion. Red cell deformation itself is usually not associated with an increase in surface area. Although the normal red cells are able to undergo a large linear extension of up to more than two-fold from their original dimensions, an increase of only 3–4% in surface area should cause the rupture of the red cells. Therefore, the maintenance of an adequate surface area:volume ratio is important to normal red cell survival [42].

In contrast, reticulocytes, which contain several organella such as mitochondria, ribosomes, and others, are much less deformable than mature red cells. During the transitional phase to mature red cells, reticulocytes reorganize the membrane phospholipids, skeletal proteins, and integral proteins to produce diskoid shapes and cell deformability. They lose transferrin receptors, insulin receptors, and fibronectin receptors. In the skeletal protein network, protein 4.2 appears at the latest stage of erythroid differentiation.

The red cells must maintain the reasonable surface area:volume ratio during their circulating life span. Reduction in surface area due to excessive membrane loss, and an increase in cell volume due to increased cell water content could give rise to a smaller surface area leading to spherocytic transformation of the red cell shape. The process creates decreased cellular deformability, by which red cell functions should be impaired and red cell survival has to be shortened [43]. Many examples are known of various membrane protein defects in clinical medicine, which are described in Chapters 9–17.

Two major types of shape changes are known, these are, echinocytic transformation and stomatocytic transformation, both of which lead ultimately to the formation of spherocytes as the final form of the red cells (Fig. 2.4). Many reagents and drugs are known to produce these changes *in vivo* and *in vitro*. Membrane proteins as well as membrane lipids are responsible for these transformations (as discussed in Sections 1.3 and 2.2). Abnormalities of skeletal proteins (spectrin, ankyrin, or protein 4.1), integral proteins (band 3, or glycophorins), and anchoring proteins (ankyrin, or protein 4.2), lead to the disease states, such as hereditary spherocytosis (Chapter 10) and hereditary elliptocytosis (Chapter 11). Abnormal red cell shape in these disorders causes early destruction of these cells by the inability to maintain their surface area and control their cell volume. Examples of abnormal red cell

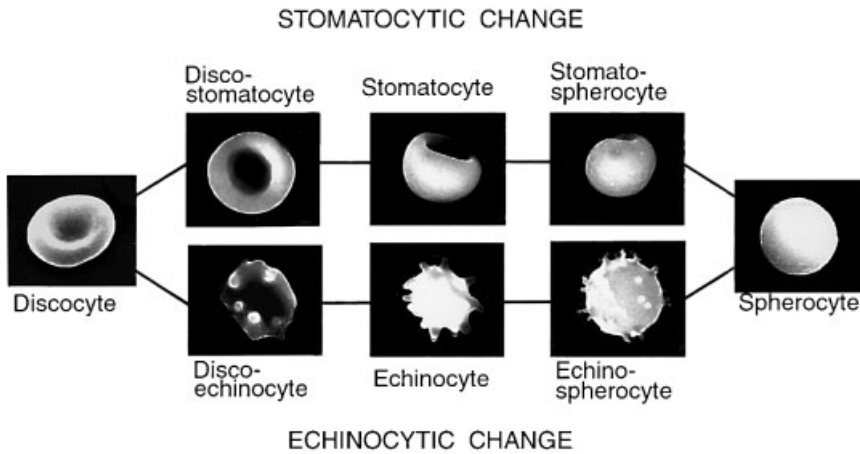


Figure 2.4 Stomatocytic and echinocytic changes as the pathogenesis of spherocytic transformation from normal discocytes.

shapes are echinocytes, stomatocytes, spherocytes, elliptocytes, acanthocytes, burr cells, schizocytes, fragmented cells, along with others.

2.3.4.2 Red Cell Deformability

The most important barrier to the circulation of red cells is the spleen *in vivo*. The red cells have to squeeze through narrow slits between the endothelial cells which line the splenic sinus wall. This procedure requires substantial deformation of the red cells [35, 37].

The term deformability means the ability of the red cells to undergo distortions and deformations and then to restore their normal shape without fragmentation or loss of integrity [42, 43].

Generally speaking, the red cell deformability is determined by three factors: (1) cell geometry, (2) viscosity of the cell contents, and (3) intrinsic viscoelastic properties of the red cell membrane. First of all, cell geometry is determined by the contribution of the cell surface area:cell volume ratio, and is clearly the most important among these three factors. The normal volume of the red cells is approximately $90 \mu\text{m}^3$, and the surface area of normal red cells with a biconcave disk shape is about $140 \mu\text{m}^2$, as discussed earlier. Therefore, the biconcave disk shape itself allows the red cells sufficient membrane and cytoskeleton for them to be significantly deformed. The red cells attain a deformability to be able to stretch as they undergo deformation and distortion under mechanical stress during their circulation. However, spherocytes or elliptocytes, which have a markedly reduced cell surface area, suffer from much less deformability. Any abnormalities of the structure of the membrane skeleton impair the normal stability and deformability of the affected red cells. Spectrins in the folded helical state are highly coiled in normal red cells [36]. The rearrangement of the cytoskeletal network, which is accompanied by

membrane deformation, is in a dynamic state: coiled and uncoiled, and extended and compressed [44]. Therefore, shape changes are not normally accompanied by a change in the cell surface area. However, when the forces of this stretching and compression are excessive, or when abnormalities of the cytoskeletal network exist, the network has to lead to membrane loss with a decrease in surface area and a change in cell geometry [45]. The best example is the poikilocytosis which is produced in microangiopathic hemolytic anemia. Many of the red cells are permanently deformed or fragmented with a plastic deformation.

The second factor, the cytoplasmic viscosity, is basically determined by the properties and the concentration of hemoglobin in the red cells. At normal intracellular hemoglobin concentrations (30 g dL^{-1} on average), viscosity does not play a major role in red cell deformability. However, when red cells become dehydrated (typically as shown in hereditary xerocytosis), red cell viscosity increases with the increased intracellular hemoglobin concentration [46].

The intrinsic viscoelastic properties of the red cells, as the third factor of the major three determinants for red cell deformability, appear to have a relatively small effect on red cell survival. A typical example is Southeast Asian ovalocytosis, where red cells carry a mutant band 3 protein [47]. Although the intact red cells and their red cell ghost membranes are extremely rigid, an almost normal survival *in vivo* is observed.

2.3.4.3 Membrane Transport and Permeability

A low potassium (5 mEq L^{-1} on average), high sodium (142 mEq L^{-1} on average), and high calcium content (5 mg dL^{-1}) are regularly observed in the plasma of normal human subjects. In contrast, normal red cells maintain their intracellular concentrations of high potassium, low sodium, and extremely low calcium, because these red cells are virtually impermeable to monovalent and divalent cations. On the other hand, the red cells are highly permeable to anions and water, which are exchanged readily. The normal red cells have at least three systems to maintain their constant volume, as follows: (1) energy-dependent active membrane pumps, (2) gradient-driven passive transporters, and (3) various channels [48].

Energy-dependent active membrane pumps Two adenosine triphosphatase (ATPase)-dependent cation pumps are known to maintain low sodium, low calcium, and high potassium levels inside the red cells [49, 50]. The first one is the ouabain-inhibitable $\text{Na}^+\text{-K}^+\text{-ATPase}$, which extrudes sodium from the cells to an extracellular medium in exchange for potassium in a 3:2 stoichiometry, a sodium pump. The second one is Mg^{2+} -dependent $\text{Ca}^{2+}\text{-ATPase}$, which is a calmodulin-activated pump [51]. This pump extrudes calcium from the red cells and succeeds in maintaining an extremely low calcium concentration inside the red cells. This function is crucial for ensuring the integrity and normal survival of red cells, since calcium is well known to demonstrate various hazardous effects on cellular functions, such as membrane vesiculation, the formation of echinocytes, calpain activation, membrane proteolysis, cellular dehydration, and decreased red cell deformability. It is

interesting to note that adenosine nucleotides (such as ATP), guanosine nucleotides (such as GTP), and most of the glycolytic intermediates, except for phosphoenolpyruvate, are impermeable through normal red cell membranes [52]. Glucose, which is the most important energy source for the intact red cells, is transported instantaneously without the expenditure of energy, through utilizing a transporter [53, 54].

Gradient-driven, passive transporters In this category, the K^+Cl^- -cotransporter, the anion exchanger (band 3), the $\text{Na}^+-\text{K}^+\text{Cl}^-$ -cotransporter, and the Na^+-H^+ -exchanger are known [55–57]. They share the common feature with each other of being able to move ions across the red cell membranes by utilizing the Na^+/K^+ gradient, which is obtained through the sodium pump. Among them, the K^+Cl^- -cotransporter is an important carrier-mediated cotransporter, especially at the stage of reticulocytes. This system is activated under conditions of cell swelling, depleted magnesium content in the cells, thiol oxidation, and acidification. In contrast, the role of the $\text{Na}^+-\text{K}^+\text{Cl}^-$ -cotransporter appears to be limited in the red cells. The Na^+-H^+ -exchanger plays an important role particularly in early erythroid maturation. A critical contribution of band 3 to anion transport is discussed in Section 5.1.2.

Various channels In the red cells, there are water channels (the aquaporins), the Ca^{2+} -activated K^+ -channel, and voltage-gated channels, which are mediated by Na^+K^+ -ATPase. As the aquaporins are membrane channel proteins they play a role as the selective pores for water crossing through the plasma membranes. Aquaporin-1 (AQP1) in the red cells along with other tissue cells ensures the red cells are able to adjust to rapid changes in osmolarity. Aquaporin was designated previously as “CHIP28”, which means a channel-like integral protein of 28 kDa [58, 59]. The hydropathy analysis revealed six highly hydrophobic membrane segments. Both the NH_2 - and the COOH -termini are exposed in the cytoplasm, and they have two external potential glycosylation sites. The number of channel molecules is approximately 200 000 copies per red cell. Although AQP1 deficient subjects exhibited no overt clinical abnormality, the AQP1-targeted knock-out mice showed hyperosmolarity after fluid restriction [60, 61]. The Ca^{2+} -activated K^+ -channel (the *Gardos* channel) is known to cause loss of K^+ in response to an increased Ca^{2+} concentration in the red cells [44, 48]. This channel is regulated by a cytoplasmic calpromotin and cyclic adenosine monophosphate, and inhibited by charybdotoxin, which is an insect toxin.

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3

Stereotactic Structure of Red Cell Membranes

3.1

Historical Background to Membrane Models

Elucidation of the pathogenesises of red cell membrane disorders requires a different approach to those for red cell enzymopathies or hemoglobin abnormalities. In the last cases, the localization of these molecules is strictly limited to the cytosol of the red cells. Therefore, scientific research is restricted mainly to the biochemistry of these proteins and the genetics of their related genes.

In contrast, in red cell membrane disorders, the investigations are broad ranging, covering the expression of membrane-related genes, the biochemistry of the expressed membrane proteins in the cytosol, the mechanism of incorporation of the expressed membrane proteins into the membranes, and the ultrastructure of red cell membranes, which are composed of a membrane lipid bilayer together with various membrane proteins. The most important key-point is that an identified membrane protein should exhibit its physiological functions in the membrane ultrastructure. Thus, molecular studies on a membrane protein identified by electron microscopy are crucial to elucidating the exact phenotype in the ultrastructure in addition to clarification of its genotype in disease states.

Historically, the first membrane model was proposed by Gorter and Grendel (1925) [1], and this was based on the experimental evidence that the surface area of membrane lipids extracted from red cell ghosts was two-fold larger than that of intact red cells. This hypothesis became one of the basic models for the structure of red cell membranes [2, 3].

The first observations on the plasma membrane of many types of cells, including red cells, was made by thin-section electron microscopy (TEM). It gave a characteristic three-layered appearance (Fig. 3.1). The electron micrograph demonstrates that the edge-to-edge thickness is 7.0–7.5 nm with an approximately 3.5 nm wide central lucent zone, which is sandwiched between two electron-dense lines, each about 2 nm wide. Robertson (1959) [4] named this structure as the “unit membrane”, in which all three layers of the 7.0–7.5 nm structure are components of one membrane. In his hypothesis, he proposed that the central electron-lucent zone corresponds to a continuous lipid bilayer, and that the two electron-dense zones represent non-lipid layers, presumably proteins and carbohydrates. For

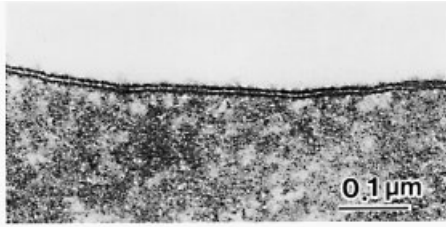


Figure 3.1 Transmission electron micrograph of membrane bilayer in normal red cells.

this model, the paucimolecular theory was utilized, which had been proposed by Davson and Danielli (1935), as reviewed in [3].

However, it became obvious that synthetic lipid membranes, biological membranes composed of a combination of lipids and proteins, and furthermore lipid-depleted membranes, all yielded a similar three-layered appearance with TEM. Thus, this area of research alone did not provide strong evidence for or against the existence of a lipid bilayer in the membrane, although the bilayer concept did receive strong support from other physical evidence such as X-ray diffraction (Engelman, 1970) [5] and differential calorimetry (Steim et al., 1969) [6].

In 1972, Singer and Nicolson proposed their new concept of the “fluid-mosaic model” [7] on the basis of the Danielli–Davson–Robertson models, which took into account thermodynamic considerations [8]. They introduced two terms to describe proteins associated with the membrane lipid bilayer, these are, integral proteins and peripheral proteins. The integral proteins are amphipathic proteins with an ionic exterior segment in contact with water at the external surface of the membrane, and a hydrophobic interior segment embedded in the lipid bilayers (Fig. 2.2). The peripheral proteins are at the surface of the membrane, and do not intercalate with the lipid matrix of the membrane. Proteins might exist either singly or as subunit aggregates. This membrane structure model was proven through the isolation of the peripheral and integral proteins. The integral proteins were able to be visualized as the intramembrane particles (IMPs) by EM with the freeze fracture method [9, 10]. Biophysical methods, such as the fluorescence recovery after the photobleaching (FRAP) method [11], enabled the direct study of the biophysical properties of the integral proteins to be made.

With the expansion and improvement of this fluid-mosaic model, the cytoskeletal network, which lies just beneath the inner leaflet of the lipid bilayer of cell membranes, was also introduced (Fig. 2.2) by Lux [12], and this added functional importance to the model.

As the asymmetry of the distribution of membrane lipids became evident, a new concept, “the micro-domain”, was incorporated into this model. Acidic phospholipids (PS and PA) interact with myeline-basic proteins and calcium resulting in the formation of the microdomain, which regulates the functions of membrane proteins [13, 14]. The structure and biological functions of various micro-domains have been investigated extensively.

3.2

Ultrastructure of Red Cell Membranes

3.2.1

Introduction

Great progress has been made in studies of red cell membrane disorders in the areas of biochemistry, biophysics, and gene analysis. Visualization of these findings has become more important in order to verify any abnormalities [15, 16]. In addition, electron microscopic findings can provide precise qualitative information as well as a great deal of insight into further studies using biochemical and genetic methods. This is especially relevant to membrane research, in which the understanding of topographical structure *in situ* is crucial (Fig. 2.2).

The red cell membranes are composed of a lipid bilayer and a cytoskeletal network beneath it. This cytoskeletal network chiefly consists of cytoskeletal proteins; spectrins, actin, and protein 4.1. The assembled cytoskeletal network is bound to integral proteins via anchor proteins [17–19].

3.2.2

Evaluation of the Cytoskeletal Network

The red cell membrane is composed of three major components: integral proteins (band 3, glycoporphins, etc.), skeletal proteins (spectrins, protein 4.1, actin, etc.), and anchoring proteins (ankyrin, protein 4.2, etc.). The skeletal network is basically composed of spectrin, protein 4.1, and actin, with other accessory proteins (tropomyosin, tropomodulin, adducin, and band 4.9).

3.2.2.1 Electron Microscopy With the Negative Staining Method

The skeletal network in the intact red cell membrane *in situ* has been shown to be a dense, sweater-like meshwork completely laminating the inner leaflet of the lipid bilayer [16, 20, 21]. When the meshwork is detached from the lipid bilayer and artificially stretched, the extended skeleton has been described primarily as a hexagonal lattice. This lattice is basically composed of long spectrin filaments and junctional complexes, which are located at the center and six corners of the hexagons [16] (Fig. 3.2).

The locations of these membrane proteins under this stretched condition have been identified by immunogold labeling. Ankyrin and band 3 were located 80 nm from the distal end of the extended spectrin molecules [16]. These findings are in good agreement with biochemical results. Protein 4.1 was bound to the distal ends of spectrin tetramers [16]. The ternary complex of actin–spectrin–4.1 along with actin-binding proteins, including adducin, is considered to be the junctional complex [16].

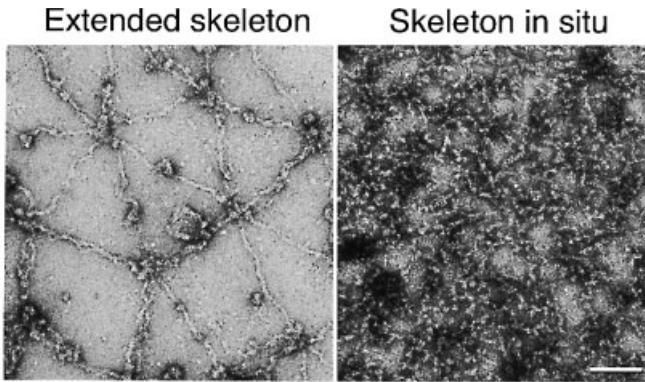


Figure 3.2
Membrane skeletons in red cell ghosts studied by electron microscopy with the shadowing method. Cytoskeletal networks in extended skeleton (left) and in an unstretched condition *in situ* (right). Courtesy by Late Professor J. Palik.

However, contrary to these observations made under artificially extended conditions, it has been shown that the basic structure of the red cell membrane *in situ* does not necessarily show a hexagonal structure.

Several trials have been carried out with electron microscopy to observe the membrane structure in red cells, using a negative staining method. The findings in these studies, however, were obtained from specimens in which the cytoskeletons had been treated with detergents, such as Triton, or trypsin. Therefore, the networks were artificially over-extended and over-stretched. These specimens appear to be adequate for identifying the exact binding sites in each membrane protein, but are probably inadequate for examination of the exact membrane structure *in situ* in normal and abnormal conditions.

3.2.2.2 Electron Microscopy With the Quick-Freeze Deep-Etching (QFDE) Method

It is now well established that the membrane in normal subjects is composed of numerous basic units resembling cages, with filaments in a three-dimensional folded configuration. When examined by electron microscopy (EM) with the quick-freeze deep-etching (QFDE) method, the skeletal network in normal red cells shows a fairly uniform distribution of filamentous structures and also uniformity of apparent branchpoints of the filamentous elements in an essentially orderly fashion [22–24] (Fig. 1.7).

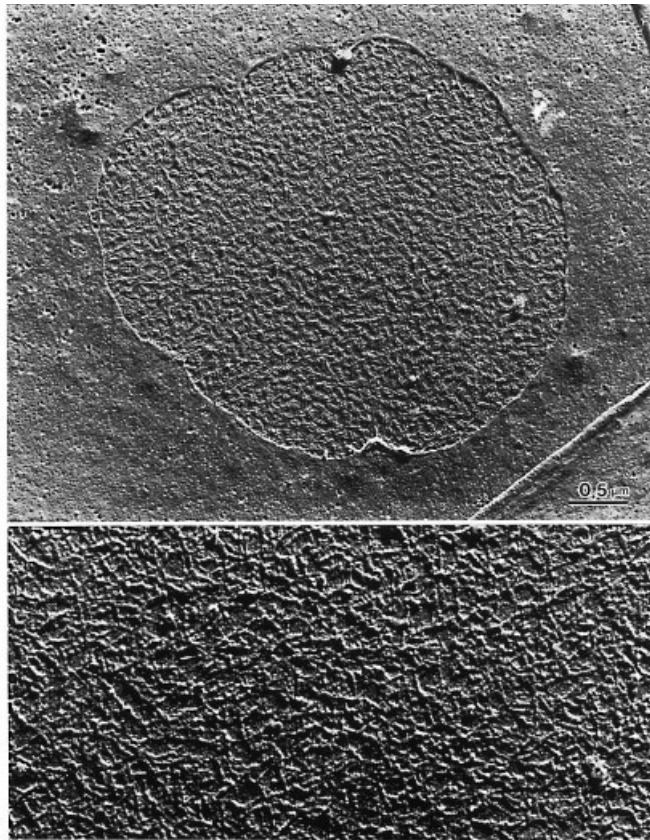
Normal white ghosts were subjected to quick-freezing, and then to deep-etching. The replicas were prepared with a platinum/carbon coating. This procedure showed that the filaments of the intact cytoskeleton existed in multi-stereotactic dimensions rather than in a single plane. The filaments coated with platinum were 48 ± 9 nm in length and 7 ± 1 nm in diameter, and they appeared to be in a folded conformation, as previously reported by Ursitti et al. [23]. A complete view of the cytoskeletal network showed it to be composed of numerous smaller basic units, the size of which, on average, was 54 ± 14 nm at the longer axis, and 23 ± 5 nm at the shorter axis [24].

3.2.2.3 Electron Microscopy With the Surface Replica (SR) Method

Although the quick-freeze deep-etching (QFDE) method appears to be the best method at the present time, it requires specialist skills and equipment, and consistently good results are not easy to obtain. In contrast, the surface replica method has been utilized widely in many fields other than the field of red cell membrane research. Consistent results can be obtained easily with this method [25] (Fig. 3.3). The immunogold method has recently been used in EM with the surface replica (SR) method, which is equally useful for examining the skeletal units. With this technique, utilizing immunogold labeling with anti-membrane protein antibodies, it has become easy to identify the exact location of major membrane proteins in a native state *in situ* [24, 25].

Therefore, regarding immunoelectron microscopy, the surface replica method is even more useful than the QFDE method, since the immunogold method is readily applicable using the surface replica method for identification of the specific components of the cytoskeletal network with specific antibodies. By the QFDE method, specimens of red cell membrane ghosts must be frozen instantaneously to -196°C . As a result, immunocytochemistry is virtually impossible. By the surface

Figure 3.3
Cytoskeletal network studied by electron microscopy with the surface replica method. Through a window of the red cell membrane structure, the cytoskeletal network is observed at the cytoplasmic side of another face of the membrane structure in the upper panel. A greater magnification demonstrates an orderly cobblestone-like pattern as shown in the lower panel.



replica method, however, immunocytochemistry is feasible, because the red cell membrane ghosts are treated at 4 °C throughout the procedure.

Under electron microscopy with the surface replica method, the cytoskeletal meshwork was composed of multiple smaller basic units, which were connected to each other. Electron microscopy using the surface replica method revealed an orderly cobblestone-like pattern in normal subjects. The basic cytoskeletal units were fairly extended with thinner, evenly stretched fiber filaments with well-organized junctional units. The basic cytoskeletal units can be categorized into four sizes: small (S) (12.5–25.0 nm in diameter along the shorter axis of each basic cytoskeletal unit), regular (R) (25.0–50.0 nm), medium (M) (50.0–75.0 nm), and large (L) (larger than 75.0 nm). In normal subjects, the size distributions of the units are $6 \pm 3\%$, $52 \pm 5\%$, $31 \pm 4\%$, and $11 \pm 3\%$, respectively [24]. In making these determinations, the diameters along the shorter axis of each basic cytoskeletal unit were selected for sizing, for consistency. The diameters along the longer axis of each basic cytoskeletal unit were also measured and the results indicated essentially the same tendency as the size distributions based on the diameters along the shorter axis of each basic cytoskeletal unit, although there were slightly greater variations in the diameters along the longer axis than those along the shorter axis.

The cytoskeletal units were basically composed of thinner filaments. These filaments were 63 ± 17 nm long and 12 ± 4 nm wide, and knob-like structures, which were attached to the longer, thinner filaments, were also observed.

The filaments in the basic units of the cytoskeletal network were identified as spectrins by immunogold labeling with anti-human spectrin rabbit polyclonal antibody, by using an electron microscope (EM) with the surface replica method [25] (Fig. 3.4). The antibody recognized the fibrous components, which were seen using the EM with the surface replica method, as spectrins. This method is also useful to detect quantitative and qualitative abnormalities of cytoskeletal network in various disease states (Fig. 3.5).

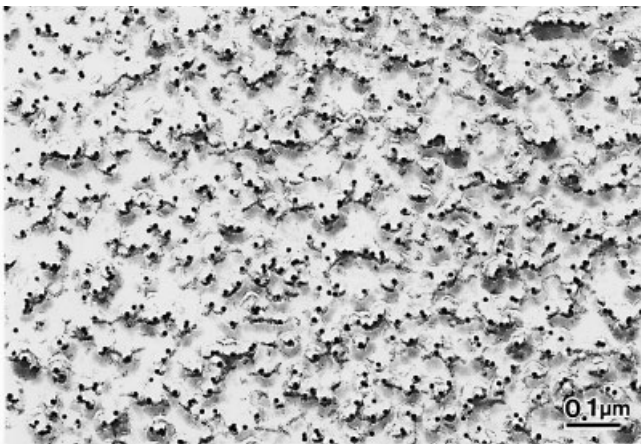


Figure 3.4 Immuno-gold labeling of spectrins in normal red cell ghosts. Electron microscopy was performed using the surface replica method with immunogold labeling by anti-human spectrin rabbit polyclonal antibody.

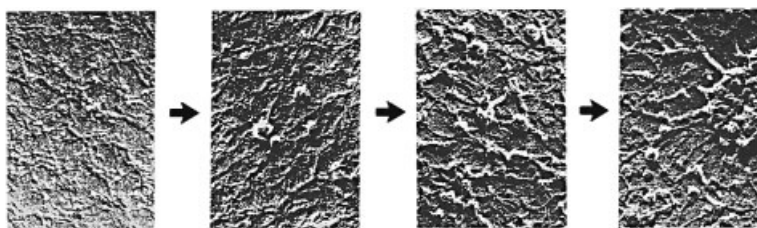


Figure 3.5 Qualitative and quantitative evaluation of cytoskeletal abnormalities by electron microscopy with the surface replica method. Normal cytoskeleton (far left) becomes deranged and distorted to the extreme

abnormality (far right) with the appearance of virtually round aggregates and with aggregates appearing like silver thistles including a centripetal arrangement of elongated, markedly stretched fiber filaments.

3.2.3

Integral Proteins Examined by Electron Microscopy With the Freeze Fracture Method

Band 3, glycophorins (A–E), and other minor membrane proteins have been identified as the integral proteins [17–19]. Among them, band 3 protein is a major integral protein with 1 000 000 copies in a human red cell, compared with approximately 200 000 copies of glycophorins. The band 3 protein is present mainly in a dimeric form.

Electron microscopically, these integral proteins are visualized as intramembrane particles (IMPs) on the P face using the freeze fracture method [24, 26] (Fig. 1.6).

In normal red cells, the number of IMPs at the inner (so-called “P”) face is $5390 \pm 420 \mu\text{m}^{-2}$, most ($71 \pm 8\%$) of which are basically small (4 to 8 nm), with $27 \pm 3\%$ of medium size (9–20 nm), and $2 \pm 1\%$ of a large size (>21 nm).

IMPs are normally distributed like clustered icebergs with irregularly open channels of water between them. This characteristic distribution pattern is mostly dependent on the function of band 3 molecules, two-thirds of which are bound to the skeletal network chiefly via ankyrin [11]. Therefore, the distribution pattern of the IMPs reflects the molecular condition of the cytoskeletal network, directly or indirectly.

To detect the abnormalities of the integral proteins by EM, the primary consideration is the quantitative evaluation of IMPs (Fig. 3.6). A typical example is the total deficiency of band 3 molecules [27]. In this disorder, the number of IMPs is decreased by 70% ($1856 \pm 226 \mu\text{m}^{-2}$) compared with that of normal subjects ($5373 \pm 292 \mu\text{m}^{-2}$) in which band 3 is present as a major constituent of the IMPs. The IMP present in the “P” face in the proband appears to be some intramembrane components rather than band 3, that is, various glycoproteins, such as glycophorins, gp 155, and minor integral proteins.

The second consideration of the abnormalities of IMPs is the sizing of these particles. As described above, approximately 70% of IMPs are small (4 to 8 nm) in size. However, in some disorders, such as total deficiency of protein 4.2, there is a marked shift to IMPs of a large size, associated with a decreased number of

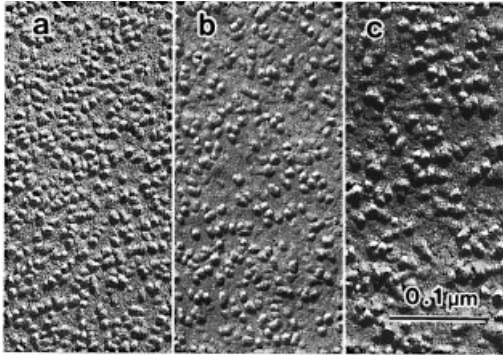


Figure 3.6 Electron micrographs of intramembrane particles (IMPs) by electron microscopy with the freeze fracture method. The numbers, sizes, and distribution patterns of the IMPs are perfectly intact in normal red cells (a). In abnormal conditions, however, the IMPs can be markedly reduced quantitatively (b), or they can demonstrate striking clustering and uneven sizing probably due to increased oligomerization (c).

IMPs. These findings strongly suggest increased oligomerization of the IMPs in this disorder [28] (Fig. 3.6).

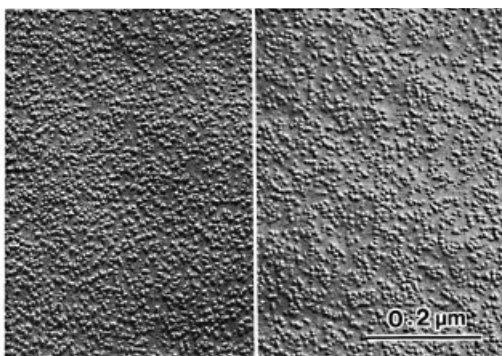
The significant contribution of protein 4.2 to the biophysical properties of band 3 was proved by utilizing inside-out vesicles (IOVs) of the normal controls and those of protein 4.2 deficiency. In the protein 4.2 deficiency, the distribution pattern of IMPs was totally deranged in IOVs, which were prepared from red cell ghosts of protein 4.2 deficiency, compared with those in the normal controls. When spectrins and membrane proteins other than band 3 were stripped from the IOVs at pH 11 in the normal controls, this experimentally produced protein 4.2 deficiency demonstrated a markedly abnormal aggregation of band 3, which was the same as that in the protein 4.2-deficient patients [28].

The third consideration is the distribution pattern of these IMPs, which are distributed randomly just like floating icebergs in the Antarctic ocean in the normal condition. It has been reported that band 3 consists of a mobile fraction (one third) and an immobile fraction (two thirds), which is fixed to the skeletal network mostly by ankyrin [11]. In normal subjects, approximately 80% of each area at the inner (so-called “P”) face contained 6 to 11 IMPs per 33 nm^2 . Only $2.4 \pm 1.0\%$ of the membrane areas contained 0 to 3 IMPs per 33 nm^2 .

However, a condition involving the marked disruption of the skeletal network with clustering of spectrin and ankyrin is expected to affect readily the state of the distribution of band 3, resulting in an abnormal distribution pattern of IMPs. This occurred in the total deficiency of protein 4.1 [protein 4.1 (–) Madrid] [24] (Fig. 3.7).

When examined using a smaller scale (33 nm^2), some areas of membrane should contain the clustered IMPs, which should be composed mainly of the immobile band 3 attached to the distorted skeletal network and/or of the mobile band 3 trapped in collapsed compartments of the skeletal proteins. However, other areas should contain a much smaller number of IMPs. In the protein 4.1 (–) Madrid, $11.7 \pm 2.5\%$ of the membrane P face contained 0 to 3 IMPs per 33 nm^2 . This abnormal distribution pattern of the IMPs in the protein 4.1 (–) Madrid case, therefore, appears mainly to reflect the markedly impaired skeletal disruption.

Figure 3.7 Abnormal distribution of IMPs detected by electron microscopy with the freeze fracture method. The distribution of IMPs can also be pathognomonic (right), even though the numbers and sizes of the IMPs are completely unaffected compared with those in the normal subject (left).



3.2.4

Visualization of Glycophorins by Field Emission Scanning Electron Microscopy

Although the functions of glycophorins (GP) have long been sought, their direct ultrastructure on the red cell membrane has never been demonstrated precisely. Through recent progress in new technology with field emission scanning electron microscopy (FE-SEM), glycophorins on human red cell membrane surfaces *in situ* have now been visualized [29]. Intact red cells were placed on the cover slip and subjected to FE-SEM (JSM-6340F, JEOL, Tokyo, Japan) at 15.0 kV, with a working distance of 8 mm, a magnification of 15 000–60 000 with the immunogold (10–20 nm in diameter) method with monoclonal anti-glycophorin A antibody. FE-SEM combined with electron microscopy with the immunofracture method utilizing anti-human glycophorin A monoclonal antibody (OSK 4-1) demonstrated approximately 3000–4000 immunogold particles on the surface of normal red cells (Fig. 3.8). No particles were detected in the En (a-) red cells. Therefore, this method is extremely specific for the detection of molecular abnormality. By FE-SEM with anti-human glycophorin A+B monoclonal antibody (GP-16: OSK-291), approximately 4000 immunogold particles were detected on the surface of normal red cells. However, around 1500 particles were present in En (a-) red cells, in which the presence of unaffected glycophorin B molecules are indicated (Fig. 3.9). Electron microscopy with the surface replica method utilizing anti-human glycophorin A+B monoclonal antibody (GP-16: OSK 29-1) demonstrated approximately 600 immunogold particles per μm^2 of the surface of normal red cells (Fig. 3.10). The size and distribution pattern were readily evaluated qualitatively and also quantitatively. Immunogold particles on the E face when using electron microscopy with the freeze-fracture method were also observed in normal red cells ($45 \pm 5 \mu\text{m}^{-2}$) with anti-human glycophorin A+B monoclonal antibody (GP-16: OSK 29-1). Abnormalities of glycophorin A, that is, a complete glycophorin A deficiency (En^{a-}), a combined deficiency of glycophorin A and glycophorin B (M^KM^K), and a Miltenberger V (Mil-V) anomaly with a complete deficiency and

a hybrid of glycoprotein A and glycoprotein B were clearly identified by this procedure. Therefore FE-SEM is a novel and definitely useful tool for studies on the abnormalities of glycoproteins.

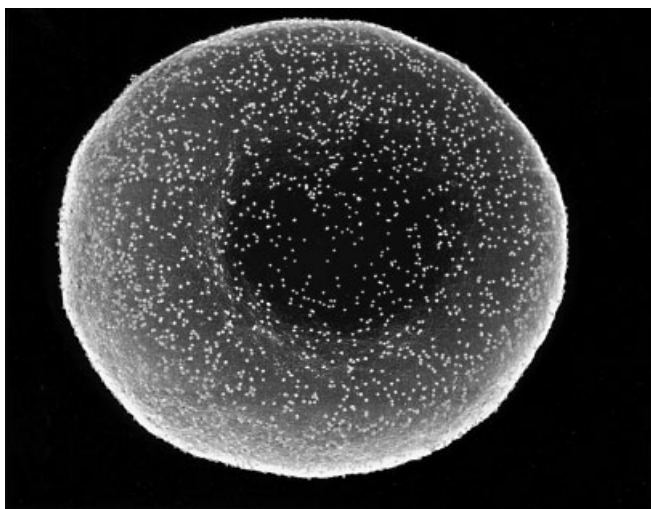


Figure 3.8
Demonstration of glycoproteins A and B by field emission scanning electron microscopy (FE-SEM) with anti-human glycoproteins A and B antibodies.

Antibodies were supplied from Dr. Tani and Ms. Senoo and Takahashi (the Osaka Red Cross Blood Center): Figs. 3.8–3.10.

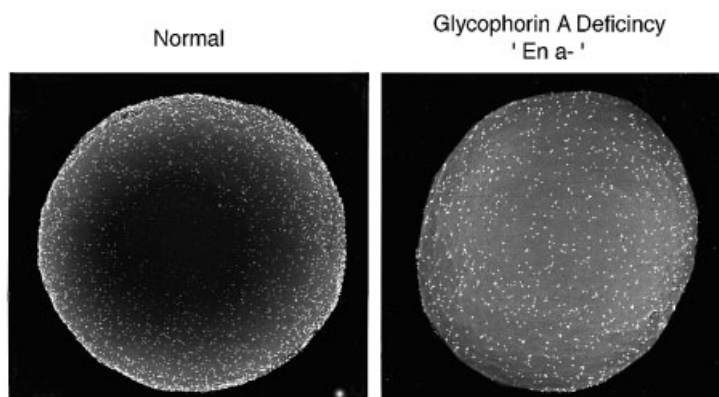
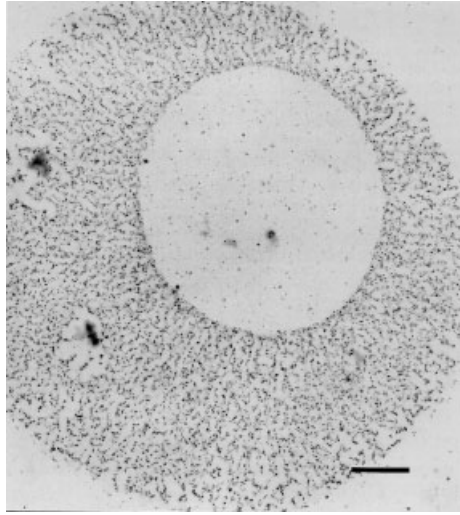


Figure 3.9 Glycophorin A deficiency [En (a—)] studied by field emission scanning electron microscopy with anti-human glycoproteins A and B antibodies. Since glycoprotein A is totally

absent, the number of particles is markedly reduced, although glycoprotein B is normally present in this patient.

Figure 3.10 Detection of glycoporphins A and B molecules by electron microscopy with the surface replica method utilizing anti-human glycoporphins A and B antibodies.



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4

Skeletal Proteins

4.1

α - and β -Spectrins

4.1.1

Introduction

Spectrins are the most abundant and largest proteins of red cell membrane skeletal proteins (Tables 1.1 and 1.2). They constitute approximately 25–30% of the total membrane proteins or 75% of the membrane skeletal proteins, and are present at a concentration of about 200 000 copies per red cell [1–4].

Spectrins are composed of two subunits, the α -chain and the β -chain, which are thus called α -spectrin (2429 amino acids, about 280 kDa) and β -spectrin (2137 amino acids, about 246 kDa) (Fig. 4.1). The number of copies of both α - and β -spectrins is 240×10^3 per red cell. The α -spectrin gene (SPTA 1) [5–7] is located on chromosome 1 (1q22–q23), and the β -spectrin gene (SPTB) [8–10] is on 14q23–q24.2. They are present as a heterodimer ($\alpha_2\beta_2$). The α - and β -spectrins are structurally related, but functionally distinct [11]. The α - and β -chains are

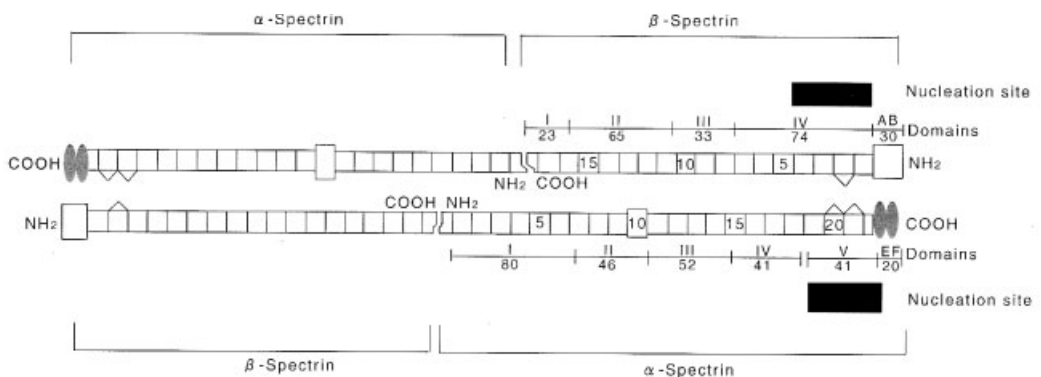


Figure 4.1 Molecular structure of α - and β -spectrins. Heterotetramers of α - and β -spectrins are arranged in an anti-parallel fashion. The head-to-head interaction and the side-by-

side interaction (at the nucleation site) are shown in this figure. α -Spectrin is composed of five domains (I to V), and β -spectrin is composed of four domains (I to IV).

aligned side by side in an antiparallel arrangement with respect to their N- and C-terminal ends. Electron microscopically, the spectrin molecule appears to have a slender and twisted rod-like structure (Figs. 1.7, and 3.3). The total length of a spectrin molecule is about 100 nm, when examined in its extended state under an electron microscope with the negative staining method (see Section 3.2.2.1). Spectrins are highly flexible to enable a variety of shape changes. Therefore, spectrins are considered as one of the major determinants of cell shape. Although the theoretical length of spectrin tetramers is about 200 nm, the actual end to end distance is about 76 nm, implying that spectrin tetramers are tightly coiled in the native ultrastructure [12]. These coiled spectrin tetramers are able to extend reversibly to a relaxed state when the membrane is stretched artificially.

4.1.2

Structure of Red Cell Spectrins

α -Spectrin (280 kDa) can be identified on sodium dodecyl sulfate (SDS)–polyacrylamide gels as a 240 kDa polypeptide [1]. The NH_2 -terminal end with an isolated, unpaired helix (helix C) is a self-association site with the COOH -terminal end of the β -spectrin molecule. Nine typical 106 amino acid repeats, which are conformation segments 1 to 9, follow after this self-association site [1–4]. There is a short central segment which lacks homology with the above-mentioned repeats, but is known to be related to SH3 domains as segment 10 [11]. A further 12 more repeats (segment 11 to 22) follow until the COOH -terminal end is reached, where two EF hand structures are present, which are involved in Ca^{2+} -binding and in regulating Ca^{2+} action in α -actinin and fodrin. However, the exact role of the SH3 domain and EF hand structures in human red cells *in vivo* are not known. The α -spectrin chain is divided into five domains, based on the results from limited tryptic digestion of α -spectrin, and designated αI (conformational segments 1 to 6: 80 kDa), αII (7 to 10 including SH3 segment: 46 kDa), αIII (11 to 14: 52 kDa), αIV (15 to 17: 41 kDa), and αV (18 to 22: 41 kDa). This method is useful for isolating and characterizing functional domains of normal and mutated spectrins in disease states, especially hereditary elliptocytosis and hereditary pyropoikilocytosis.

β -Spectrin (246 kDa) is isolated as a 220 kDa polypeptide by SDS–polyacrylamide gel electrophoresis (PAGE) [1]. The structure of β -spectrin begins with a non-homologous NH_2 -terminal end that contains an actin-binding domain, followed by 17 homologous 106 amino acid repeat segments [1–4]. Subsequently there is the putative protein 4.1-binding site [13] and a short nonhomologous COOH terminal segment with a consensus sequence for at least four phosphorylation sites for casein kinase 1 [14]. The state of phosphorylation is related to membrane mechanical stability, since it is known that increased phosphorylation decreases membrane stability, and also that decreased phosphorylation increases it [15]. Functionally, repeat 15 and part of repeat 16 are arranged in a β sheet structure and form the binding site for ankyrin [16]. β -Spectrin is also divided into four domains, designated as βIV (conformational segments 1 to 7: 74 kDa), βIII (8 to 10: 33 kDa), βII (11 to 16: 65 kDa), and βI (17 down to the COOH end: 23 kDa). In the β -spectrin, no SH3

segment is present. Nucleation sites of α -spectrin and β -spectrin are in α V (conformation segment 19 to 22), and in β IV (1 to 4), respectively.

The presence of repeats in α - and β -spectrins strongly suggests that spectrin evolved from the duplication of a single ancestral gene [5–10]. The homologous 106 amino acid repeats in α - and β -spectrin fold into α -helical segments containing three antiparallel helices which are connected by short nonhelical segments. Each repeat has a triple-helical structure that is about 5 nm long and 2 nm wide. The unit is rotated 60° (right-handed) relative to the neighboring repeats [17, 18].

Each repeat is composed of three helices, i. e., helix A, helix B, and helix C. The α -helix (helix A) with 28 amino acids in a straight arrangement reverses itself and forms the next α -helix (helix B), which is 34 amino acids long. This is followed by another reverse turn and the third 31 amino acid α -helix (helix C), which bends in the middle. These three helices are in a triangular array and are bound together by both hydrophobic and electrostatic interactions. Hydrophobic amino acids are aligned on one face of each helix, and are spaced every third or fourth residue, because an α -helix makes one turn every 3.6 residues. Additional salt bonds are present between the mostly polar amino acids, particularly between helices A and C, and B and C. The three helices are tilted away from each other by 10° to 20°, so that the COOH-terminal end of each repeat is wider than the NH₂-terminal end. This molecular arrangement enables the following repeat to be attached without any change to the structure. The repeats connect to each other through the helices A and C, forming one long α -helix. Helix B of the proximal repeat overlaps helix A of the distal repeat, because the connection is tight. Interactions between the two helices appear to restrict the mobility of the repeats at the repeat junction.

Several residues appear to be highly conserved in the repeat segments, such as tryptophan at position 45, leucine at position 26, arginine at 22, aspartate at 38, aspartate or glutamate at 41, lysine at 71, histidine at 101, and hydrophobic amino acids at 1, 12, 15, 26, 35, 46, and 68, respectively [11].

Although the presence of homologous 106 amino acid repeats suggests that spectrin evolved from duplication of a single ancestral minigene, the genomic organization of both α - and β -spectrin genes in human beings reveals that the size and position of exons does not necessarily correspond to the structural or conformational unit of spectrin repeats.

4.1.3

Functions of Red Cell Spectrins

Spectrins are the key protein for the composition of the cytoskeletal network, which regulates cell shape, membrane deformability and stability, and the lateral mobility of band 3 as an integral protein [1–4]. Spectrin has spring-like properties [19]. These important functions of spectrins are mainly mediated by self-association of the spectrins themselves.

Spectrin heterodimers ($\alpha\beta$) associate to form spectrin heterotetramers ($\alpha_2\beta_2$) and higher oligomers (Fig. 4.2). On the membrane, tetramer ($\alpha_2\beta_2$) is present predominantly. Spectrins stay as the tetramer at a physiological ionic strength and low-tem-

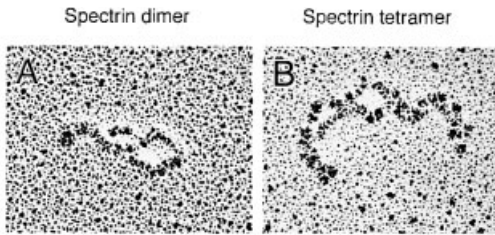


Figure 4.2 Demonstration of spectrin dimer (A) and spectrin tetramer (B) by electron microscopy with the shadowing method. By courtesy of the late Professor Jiri Palek.

perature (25 °C), whereas they dissociate into dimers at low ionic strength and 37 °C. The equilibrium is kinetically frozen at 0 °C. In normal red cell ghosts, approximately 5 % of the extracted spectrin is in the dimeric form and about 50 % is in the tetrameric form. The remainder is the spectrin oligomers and the protein complexes of spectrins, actin, protein 4.1, and dematin, which have very high molecular weights. This procedure has been utilized for elucidating molecular abnormalities of spectrins in the disease states, especially hereditary elliptocytosis.

For the self-association of spectrins, two mechanisms have been considered: (a) the head-to-head interaction of the spectrins, and (b) their side-by-side interaction (Fig. 4.3). For the first mechanism, the interconversion of spectrin dimers to tetramers requires a reversible opening of the dimeric bond and formation of two new β attachments. The $\alpha\beta$ contact closely resembles the triple-helical structure of native spectrin repeats [19, 20]. In the contact site, two of the helices (helices A and B) at the COOH-terminal end of the β -spectrin molecule are bound to one helix (helix C) of the NH₂-terminal end of the α -spectrin molecule [20].

The second mechanism is the side-by-side association of the α - and β -spectrins, which occurs in a zipper-like process. It starts with a defined nucleation site composed of four repeats, that is, $\alpha 19$ to $\alpha 22$ from the α -spectrin, and $\beta 1$ to $\beta 4$ from the β -spectrin, respectively [21]. These repeats are located at the tail end of each molecule, to which actin binds. Two of the α repeats and one of the β repeats have eight residue insertions which participate in the interchain interaction [22]. These are located between conformational segments $\alpha 20/\alpha 21$ and $\beta 2/\beta 3$. After the initial tight

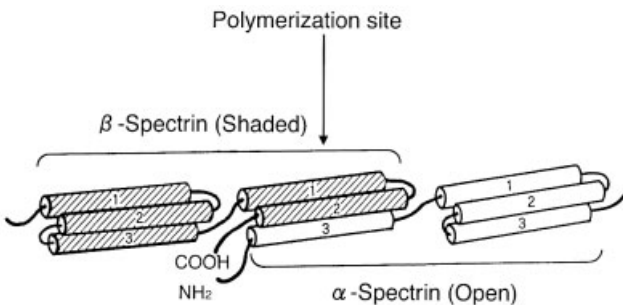


Figure 4.3 Head-to-head contact of α - and β -spectrins in normal red cells. β -Spectrins are shown as shaded helices, and α -spectrins are denoted as open helices. A polymerization site is composed of the last two helices of β -spectrins and the first one helix of α -spectrin.

association of the complementary nucleation sites, a conformational change is initiated that promotes the pairing of the remaining part of the two chains [23]. A common α -spectrin polymorphism, α^{LELY} , interferes with normal nucleation and decreases the synthesis of functionally-competent α -spectrin chains [24]. This may influence clinical expression in spectrin mutations [25].

The linkage of spectrins to other red cell membrane proteins is also functionally important. Binding of spectrins to ankyrin requires almost the entire 15th repeat segment and a small portion of the 16th repeat of the β -spectrin molecule [16]. It has been reported that β -spectrin and nonerythroid β -spectrin (β -fodrin) contain an ankyrin-independent site in the NH_2 -terminal half of each molecule, which binds to brain membranes. The binding is inhibited by Ca^{2+} /calmodulin. This site is known as the membrane association domain 1 (MAD1) [26]. β -Fodrin and the muscle/brain isoform of red cell β -spectrin have a second Ca^{2+} /calmodulin-independent site (MAD2) near to the COOH -terminal end.

Another linkage of spectrins to the membrane is mediated by association with the junctional complex that includes spectrin, actin, and protein 4.1, which form a ternary or higher-order complex by linking spectrin tetramers to one another in a tail-to-tail fashion [4]. The actin binding site is located at a region near to the NH_2 -terminal end of β -spectrin that contains a 27 amino acid sequence, which is also highly conserved in α -actinin, dystrophin, actin-binding protein (ABP-120), and others.

4.1.4

Erythroid and Nonerythroid Spectrins

There are a wide variety of spectrin-related proteins in many tissues and animal species [3, 4]. Two closely related, yet distinct mammalian α -subunits of spectrin are known, i.e.: α -spectrin in mature erythroid cells and α -fodrin in all other tissues [27]. In mammals, there are two β -subunits, i.e.: β -spectrin and β -fodrin. A third subunit (β -heavy spectrin) with a molecular weight of 430 kDa is present in *Drosophila*. β -Spectrin exists in both erythroid and muscle/brain isoforms [28–30]. Nonerythroid spectrins are basically composed of two nonidentical, high molecular weight subunits (such as α -spectrin and β -spectrin), which are composed of homologous repeat units of 106 amino acids.

Fodrin (synonyms: tissue spectrin, brain spectrin, spectrin II, or spectrin G) is a heterodimer of α - and β -fodrin chains [2]. The biological functions are essentially the same as those in red cell spectrin (spectrin I, or spectrin R). However, there are some differences between them. The α -fodrin has a calmodulin-binding site which is not present in α -spectrin. In addition, β -fodrin contains a pleckstrin homology domain which is a sequence motif in MAD2, but red cell spectrin does not.

Erythroid spectrins, nonerythroid spectrins, dystrophin and its homologues, and α -actinin are believed to belong to the so-called spectrin super-family. These proteins have the same characteristic features such as the flexible, rod-like shapes of the side-by-side arrangement of the proteins in an antiparallel fashion. They are composed of homologous amino acid repeats of 106, 109, and 120 residues

of spectrin, dystrophin, and α -actinin, respectively, which are folded into triple α -helical segments [1, 2]. Amongst these there is homology in the sequence of the triple-helical repeats with conservation of tryptophan at position 45 or 46. The composition of the repeats are homologous mainly between spectrin and dystrophin, whereas that of the actinin repeat is less homologous. The COOH-terminal regions are homologous between α -spectrin, dystrophin, and actinin, which have the potential for Ca^{2+} -binding, EF hand structures. The NH_2 -terminal regions are also homologous in β -spectrin, dystrophin, and α -actinin which demonstrate a potential actin-binding site.

Great variability exists with respect to the subcellular localization of erythroid and nonerythroid spectrins [1–4]. The spectrin superfamily proteins are essentially present in the membrane skeleton of the mammalian red cells, but are also expressed in various tissues by developmentally regulated mechanisms, such as neural development, oogenesis, epithelial cell polarity, embryogenesis, viral transformation, the IgG binding to cell surface receptors, and apoptosis. It has been shown that tissue-specific differential modification of the COOH-terminal region of β -spectrin produces various β -spectrin isoforms. The best example is a muscle isoform of β -spectrin [28]. The isoform results from alternative mRNA splicing of the mRNA transcript of the erythroid β -spectrin gene. The splicing alters translation termination near to the COOH-terminus, leading to elongation of the COOH-terminus of the muscle form. At the present time, two α -spectrin isoforms and five β -spectrin isoforms have been reported [29]. A β III isoform is localized on the Golgi apparatus in many cell types [30].

4.2

Protein 4.1

Protein 4.1 in red cells is a phosphoprotein present in 200×10^3 copies per cell [31, 32]. The cloned protein is globular (5.7 nm diameter) and has a molecular weight of 66 kDa, but migrates as a 78 or 80 kDa protein on SDS–PAGE gels. The protein 4.1 gene (EL1 or EPB41) [33–35] is located on chromosome 1p36.1 [36], which encodes 588 amino acids (Tables 1.1 and 1.2).

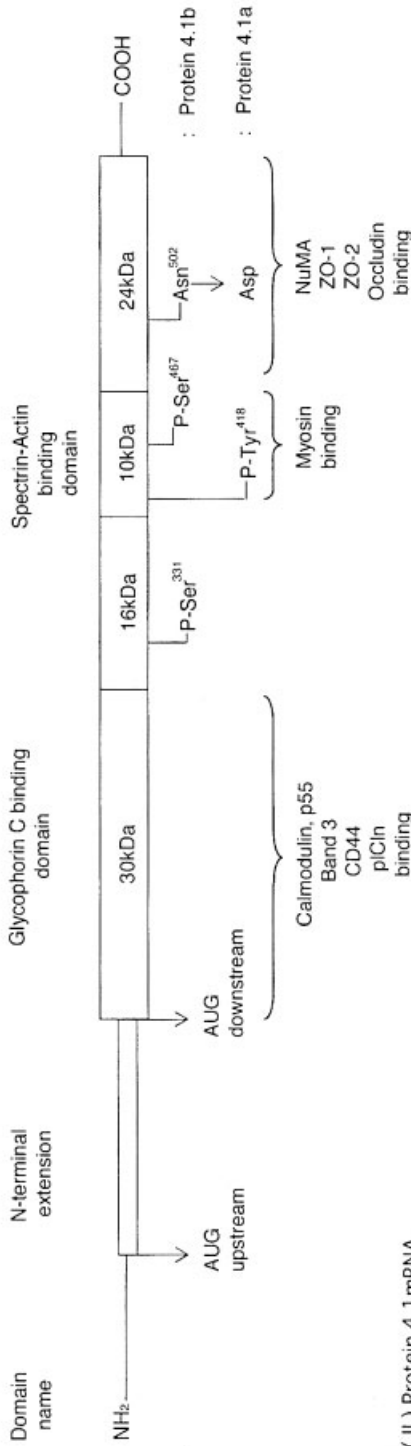
4.2.1

Structure of Protein 4.1

Chymotryptic digestion and limited sequencing demonstrate the presence of four domains of red cell protein 4.1 [32]: (1) a 30 kDa domain (residues 1 to ~300) at the NH_2 -terminal end, (2) a 16 kDa domain (residues 300 to 404), (3) a 10 kDa domain (residues 405 to 471), and (4) a 22 to 24 kDa domain (residues 472 to 622) at the COOH end (Fig. 4.4).

In red cells, there are two forms of protein 4.1 with different molecular weights, that is, protein 4.1a (80 kDa) and protein 4.1b (78 kDa). Protein 4.1a, with the higher molecular weight, results from the deamidation of two Asn residues (478

(I) Protein 4.1



(II) Protein 4.1 mRNA

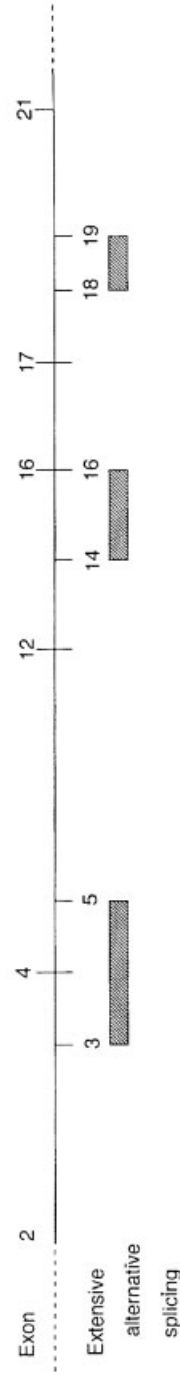


Figure 4.4 Molecular structure of protein 4.1. A schematic structure of protein 4.1 molecule is shown in (I), and the protein 4.1 mRNA is demonstrated in (II). The details are given in text.

and 502) within the 22 to 24 kDa domain in a non-enzymatic, age-dependent fashion that lowers the mobility of the protein in gels [37]. Therefore, protein 4.1a is hardly apparent in young red cells, and increases as red cells age. Thus, protein 4.1a provides a useful indicator of red cell senescence.

Considering the characteristic features of the structure of protein 4.1, clustering of cysteine residues is observed near the NH₂-terminal, and O-linked glycosylation is present in the 10 kDa domain [31]. The glycosylation contributes to its higher apparent molecular weight on polyacrylamide gels than is predicted on the basis of known amino acid sequence. In addition, the NH₂-terminus is definitely basic, whereas the COOH-terminus is clearly acidic. It has been known that red cell protein 4.1 is highly phosphorylated [31]. Phosphorylation sites are located near the COOH-terminal end of the 30 kDa domain and in the middle region of the 10 kDa domain. The latter site appears to be cyclic AMP dependent, but the others may be regulated by protein kinase C. At least one site appears to be a substrate for cdc kinase.

4.2.2

Binding to Other Membrane Proteins

The most important role of protein 4.1 is in the linkage of the spectrin–actin membrane skeleton to the lipid bilayer by facilitating complex formation between the spectrin–actin fibers [31, 38], the cytoplasmic domain of band 3 [39], p55 [40], and glycophorin C [41]. Protein 4.1 binds tightly (binding coefficient $K_d \sim 10^{-7}$ M) to β -spectrin very close to the actin-binding site [13]. For this activity, a 21 amino acid within the 10 kDa domain is critical. The interaction of protein 4.1 with spectrin and actin is blocked by protein kinase A phosphorylation at residues in Ser³³¹ in the 16 kDa domain and Ser⁴⁶⁷ in the 10 kDa domain, and also by tyrosine kinase phosphorylation at Tyr⁴¹⁸ in the 10 kDa domain [31]. The ternary complex is also regulated by Ca²⁺ and calmodulin [42]. Calmodulin binds to a site within the 30 kDa domain of protein 4.1 in a Ca²⁺-independent manner ($K_d \sim 5 \times 10^{-7}$ M).

The 30 kDa domain of erythroid protein 4.1 is involved in its binding to proteins such as p55, glycophorin C, band 3, and other embedded moieties, e. g., the chloride channel pICln [43]. Protein 4.1 binds directly to glycophorin C to a site near amino acids 82 through 98 in the tail of this molecule [41]. It also binds to a more distal portion near residues 112 through 128, by means of the protein p55 [40]. The role of the interaction between protein 4.1 and glycophorin C is well established. Protein 4.1 deficient red cells are also deficient in glycophorin C, but not glycophorin A or band 3. The residual glycophorin C that is only loosely bound to the skeleton becomes tightly bound after protein 4.1 is reconstituted. Both protein 4.1 and glycophorin C bind p55, which enhances or regulates their interaction. The mechanical weakness of glycophorin C-deficient membranes is totally restored by restoration of protein 4.1 or its 10 kDa spectrin–actin binding domain. Under the physiological condition *in vivo*, approximately 40 % of protein 4.1 is bound directly to glycophorin C, 40 % is bound indirectly through the protein p55, and 20 % is bound to band 3.

4.2.3

Extensive Alternative Splicings

It is known that protein 4.1 is extremely heterogeneous with respect to molecular weight, abundance, and intracellular localization [31]. Red cell protein 4.1 (P4.1R) isoforms come from a single genomic locus near the Rh locus at chromosome 1p36.1 [36]. Its primary mRNA transcript from the gene (250–300 kb long) is subjected to extensive alternative splicing, producing a diverse protein family through various mRNA (6.5 to 7.0 kb long) [44] (Fig. 4.5). At the present time, 12 alternatively spliced exons, as well as an important cryptic acceptor site, are known.

Protein 4.1 utilizes two different initiation codons, termed “upstream” and “downstream” initiation codons, which translate to isoforms of 135 and 85 kDa, respectively [31]. The 85 kDa isoform is created by the splicing out of a 17 nucleotide motif that contains the upstream translation initiator. In most tissues, this sequence is spliced in, and a downstream 80 base pair motif is spliced out, producing the elongated 135 kDa isoform that contains an additional 209 amino acids attached to the NH₂-terminus of the shorter isoform of protein 4.1. This is because the upstream translation start site, when present, is used almost exclusively [33]. The 85 kDa shorter isoform which is encoded by the downstream initiation codon is found primarily in red cells.

In erythroid protein 4.1 (P4.1R), splicings appear to be critical for red cell functions in only two regions, i. e., at the 10 kDa spectrin–actin binding domain, and in the 5′-untranslated region [31].

At the 10 kDa domain, three alternatively spliced exons are located at the 5′-end of this domain, which is the spectrin–actin binding domain. Exon 16, which is one of the three exons and is 63 nucleotides long, encodes for 21 amino acids at the 5′-terminus. This exon 16 is also expressed in muscle and testis [45], which are actually nonerythroid tissues. Two other exons (exons 14 and 15) are adjacent to the one responsible for binding to spectrin–actin. In red cells, the protein 4.1 mRNA contains only exon 16 out of these three exons [33, 46], whereas lymphocytes have none of the exons, and the brain retains all three exons [45]. Therefore, exon 16 appears to provide erythroid and stage-specific expression.

4.2.4

Nonerythroid Protein 4.1 Isoforms

Multiple alternative mRNA splicing events generate isoforms with different affinities for membrane and intracellular structures. The high (120–150 kDa) and low molecular weight isoforms (60–90 kDa) coexist in varying ratios in most nonerythroid tissues. Although the functional importance of the high molecular weight isoforms remains unknown, their major part of protein 4.1, which is 80 kDa upstream from the COOH-terminus, is identical to their low molecular weight analogues. Therefore, the additional amino acid residues in the high molecular weight isoforms are equivalent to a headpiece [31, 44]. It is interesting to note that high molecular weight isoforms associate with NuMa (a major organizing protein of

the mitotic apparatus) [40, 47, 48], ZO-2 (a component of the tight junctions in epithelial and endothelial cells), and other membrane associated guanylate kinase (MAGUK) family proteins, in addition to p55 [40, 49, 50]. The diverse structure of these spliced isoforms appears to provide for the multiple subcellular locations for their specific functions. Although these isoforms demonstrate their widespread expression and interaction with key components, such as mitotic spindles and tight junctions, there is a surprising fact that erythroid protein 4.1 gene-targeted knock-out mice exhibit only anemia and subtle neurological abnormalities [51].

As regards the high molecular weight isoforms, their junctional significance is still unknown [31]. The high molecular weight isoforms are completely absent from mature red cells. mRNA containing the 17 amino acid extension of exon 2, which is required for the synthesis of high molecular weight forms, is essentially absent from early erythroid precursors (younger than proerythroblasts) [33, 45]. Only the downstream translation initiation site is utilized for erythroid protein 4.1 biosynthesis during erythropoiesis.

It is now clear that protein 4.1 and its family analogues demonstrate a mosaic of sequence homologues with other proteins [52]. A typical example is the 20–45 % homology, which is observed at the NH₂-terminus of protein 4.1 family proteins. The FERM (Four. 1 protein, Ezrin, Radixin, and Moesin) domain, which is present at the NH₂-terminus, defines a family of proteins linking the cytoskeleton including actin to membranes in various cells [53, 54]. The FERM has previously been known as a family of actin binding proteins (moesin, ezrin, radixin, and merlin), which are also homologous with coraclin. This is a *Drosophila* protein that interacts with a large disk protein (Dlg) homologous with tumor suppressor proteins in mammals [55]. Erythroid protein 4.1 (P4.1R) is the prototype of a much more closely related family of at least four members, such as, P4.1 of a generally distributed type (P4.1G), that of a brain type (P4.1B), or that expressed predominantly in neuron (P4.1N). The extent of the homology among these isoforms is 60–95 % [56, 57].

4.3

Actin

Red cell actin (β -actin), which is a 43 kDa protein, is present in abundance ($400\text{--}500 \times 10^3$ copies per red cell) (Tables 1.1 and 1.2). The red cell actin content is 5.5 % (w/w) of the total red cell membrane proteins [58–60]. The β -actin gene (ACTB) is located on the chromosome 7 (7p12–p22) encoding 375 amino acids. Red cell actin is similar to other actins in its structure and functions. Although the β -actin is distributed in various nonmuscle cells including red cells, red cell actin is organized as short, double-helical F-actin protofilaments 12 to 13 monomers long and approximately 35 nm in length. Red cell actin interacts with spectrins, adducin, protein 4.1, and tropomyosin for the sake of their stability [38, 58, 61, 62]. The actin also binds to tropomodulin by capping of the slow growing or pointed end of the actin filament [63]. The state of actin polymerization is functionally important to red cell membrane flexibility, which increases when actin poly-

merization is inhibited. It is also true that increased polymerization of actin makes the red cell membrane more rigid. Spectrin dimers bind to the side of actin filaments at a site near the tail end of the spectrin molecule. On average, six spectrin ends make a complex with each actin oligomer, producing an irregular network, which is approximately hexagonal [60]. Each spectrin–actin junction is stabilized by the formation of a ternary complex with protein 4.1 [38].

4.4

Other Minor Skeletal Proteins

4.4.1

The p55 Protein

The p55 protein is a phosphoprotein member of the MAGUK (membrane-associated guanylate kinase) family of proteins [40, 58]. The protein is a 55 kDa skeletal protein, which has 80×10^3 copies per red cell (Tables 1.1 and 1.2). The p55 gene (MPP1) is located on the X chromosome (Xq28), which encodes 466 amino acids. The protein p55 is the human homolog of *dlg*, a *Drosophila* tumor suppressor gene [40]. This protein is composed of three domains: (1) an NH₂-terminal domain of unknown function that is also present in *dlg*, (2) a central SH3 domain that is embedded in the MAGUK domain, and (3) a COOH-terminal guanylate kinase domain [64, 65]. The protein appears to be present in a dimeric form, extensively palmitoylated, and tightly bound to the membrane [66]. Homologues of the p55 include signal transduction proteins, tumor suppressor genes, and proteins important in cell-to-cell interactions. This protein is expressed throughout erythroid differentiation and is widely expressed in nonerythroid tissues.

The p55 binds to the 30 kDa domain of protein 4.1 through a 39 amino acid binding motif in the COOH-terminal MAGUK domain and to the cytoplasmic tail of glycophorin C by means of its single PDZ motif. The p55 binds to the 30 kDa domain of protein 4.1 ($K_d \sim 2 \times 10^{-9}$ M) and to the cytoplasmic domain of glycophorin C ($K_d \sim 7 \times 10^{-9}$ M). In the disease states, patients who are actually deficient in either protein 4.1 or glycophorin C also lack the p55 [67].

4.4.2

Adducin

Adducin, which is a Ca²⁺/calmodulin-binding phosphoprotein, is composed of $\alpha\beta$ adducin heterodimers [68, 69]. This protein is located at the spectrin–actin junctional complex [58]. The α and β adducin are structurally similar proteins encoded by separate genes (Tables 1.1 and 1.2). A theoretical molecular weight of α -adducin, which is calculated from the results of gene sequencing, is 81 kDa, 1 % of the total red cell membrane proteins, and with 30×10^3 copies per red cell. However, the actual molecular weight of this protein is 103 kDa on the SDS-PAGE gels, which is quite larger than the theoretical one, because some other components

(such as glycosylated chains) are present on this molecule. β -Adducin (80 kDa) is 97 kDa on the SDS-PAGE gels, 1 % of the total red cell membranes proteins, and with 30×10^3 copies per red cell. The α -adducin gene (ADDA) is located on chromosome 4 (4p16.3), which encodes 737 amino acids. The β -adducin gene (ADDB) is located on chromosome 2 (2p13–2p14), which encodes 726 amino acids. A third nonerythroid adducin (γ -adducin) is also known. The subunits have three domains: (1) an NH_2 -terminal domain (39 kDa) with a globular head, (2) a 9 kDa domain of the connecting neck, and (3) a protease-sensitive domain (33 kDa) with an extended COOH-terminal tail [68, 70]. The last domain contains mainly hydrophilic residues and 22 amino acid segments homologous with the MARKS phosphorylation domain that regulates Ca^{2+} /calmodulin-regulated capping and bundling of actin filaments [71]. The four head domains cluster in a globular core, and the tail domains extend to interact with spectrins and actin. Adducin increases the binding of spectrin to actin, just like protein 4.1, whereas adducin does not interact directly with spectrin in the absence of actin, unlike protein 4.1. The tails of both α - and β -adducin bind to the actin-binding domain at the N-terminus of β -spectrin, and to the second spectrin repeat [72].

Adducin is expressed at the stage of erythroblasts, but is only incorporated into the red cell membrane structure at a late stage in erythroid development [73]. Adducin contributes to the early assembly of the spectrin–actin complex, which is regulated by phosphorylation of the COOH-terminal domain of adducin by protein kinase C [74, 75]. In this event, adducin does not bind directly to spectrin in the absence of actin. Targeted inactivation of β -adducin in mice produced compensated spherocytic anemia and neurologic abnormalities [76]. For this assembly of a spectrin–actin–adducin ternary complex, the actin-binding domain of β -spectrin and the first two spectrin repeats are required. Adducin is also present in various nonerythroid cells [68], especially at sites of cell-to-cell contact. The protein assembles at these sites in response to extracellular Ca^{2+} , and dissociates when phosphorylation of the protein is activated by protein kinase C.

4.4.3

Dematin (Protein 4.9)

Dematin has been known to be associated with actin in erythroid and nonerythroid cells [58]. Human red cell dematin consists of two chains of 48 kDa and 52 kDa, which are present in a ratio of 3 (48 kDa) to 1 (52 kDa) (Tables 1.1 and 1.2). The native protein is a trimer [77]. There are 40×10^3 trimeric copies per cell, and the content of dematin is approximately 1 % of the total red cell membrane proteins. The dematin gene (EPB 49) is located on chromosome 8 (8p21.1), which encodes 383 amino acids [77, 78]. Dematin has two binding sites for F-actin, and bundles actin filaments into cables, which is inhibited by phosphorylation with protein kinase A, but not with protein kinase C. The COOH-terminal half of the 48 kDa subunit is similar to villin, which is known as an actin-binding protein that induces growth of microvilli and reorganizes actin filaments in brush borders. The 52 kDa subunit of dematin has an additional 22 amino acid sequence

in the C-terminal domain of the 48 kDa subunit [77]. This sequence resembles that in protein 4.2 [79]. Dematin appears to attach to a lipid or integral membrane protein, since it remains associated with the membrane during the extraction of other skeletal proteins.

4.4.4

Tropomyosin

It is known that tropomyosin is also associated with actin [58]. Red cell tropomyosin is a heterodimer of 27 kDa and 29 kDa subunits on SDS–PAGE gels, on which tropomyosin migrates in the region of band 7 [80, 81]. There are 80×10^3 copies per cell, and its content is approximately 1% of the total red cell membrane proteins (Tables 1.1 and 1.2). The tropomyosin gene (TPM 3) is located on chromosome 1 (1q31), which encodes 239 amino acids. Stoichiometrically, one copy of tropomyosin binds to every six to eight actin monomers, which is just sufficient to line both grooves of the actin protofilament. The function of tropomyosin appears to be for stabilizing the short erythroid actin filaments and to help spectrin–actin interactions [82].

4.4.5

Tropomodulin

Tropomodulin is a 41 kDa protein that binds to tropomyosin in a 2:1 molar ratio with a K_d of 5×10^{-7} M [83]. Each protein binds to the NH_2 -terminal region of the other. The molecular size of tropomodulin is 43 kDa on SDS–PAGE gels [58]. There are 30×10^3 copies per cell (Tables 1.1 and 1.2). The tropomodulin gene (TMOD) is located on chromosome 9 (9q22), which encodes 359 amino acids. This protein also binds to actin. Capping is enhanced when the grooves of the actin filament are lined with tropomyosin. Tropomodulin appears to have a tight association with the membrane.

4.4.6

Other Membrane Proteins

Myosin [58, 84, 85] and caldesmon (a 71 kDa calmodulin-binding protein) [86] are known as minor components of the red cell membrane proteins, although their physiological functions have not been elucidated in detail.

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5

Integral Proteins

5.1

Band 3

Band 3 is the major integral protein of the red cells, and is also known as anion exchanger-1 (AE1) [1–4]. This protein is a transmembrane glycoprotein with a molecular mass of about 100 kDa, and comprises 25 to 30 % (w/w) of the total membrane proteins (Tables 1.1 and 1.2). There are about 1200×10^3 copies per cell. Band 3 migrates as a diffuse band on SDS–PAGE gels due to heterogeneous glycosylation [5, 6].

The human band 3 cDNA is about 4.7 kb in length and encodes a 911 amino acid polypeptide [7, 8]. The gene (EPB3) is located on chromosome 17 (17q21–q22).

Structure and functional interpretation of the cytoplasmic domain of erythrocyte membrane band 3 have recently been shown crystallographically [9].

5.1.1

Structure of Band 3

Band 3 is composed of the cytoplasmic (NH₂-terminal) domain and helices and β -sheets to form the transmembrane (COOH-terminal) domain [1–5] (Fig. 5.1).

The cytoplasmic (NH₂-terminal) domain of band 3 is composed of the first 403 amino acids. The major part of this domain (amino acids 1 to 359) is released from the membrane by treatment with chymotrypsin or trypsin as a 43 kDa fragment [10]. The two regions can be separated by chymotrypsin cleavage at the inner membrane (position 359). A second chymotryptic site is accessible at the external surface at position 553. This domain is an elongated, water-soluble, 403 amino acid segment with a flexible proline-rich hinge near the center, which is located between amino acids 175 and 190 of this domain. The first 45 amino acids in the cytoplasm are highly acidic. The remainder of the domain is fairly mobile. It extends at a high pH and contracts at a low pH [5].

The transmembrane (COOH-terminal) domain (amino acids 404 to 911), which is composed of approximately 52 kDa (17 + 35 kDa), folds into helices and β -sheets to form 12 to 14 membrane-spanning segments containing the anion transport channels. The boundary between the NH₂-terminal (cytoplasmic) tail and the first membrane-spanning segment (amino acids 400 to 404) is highly conserved

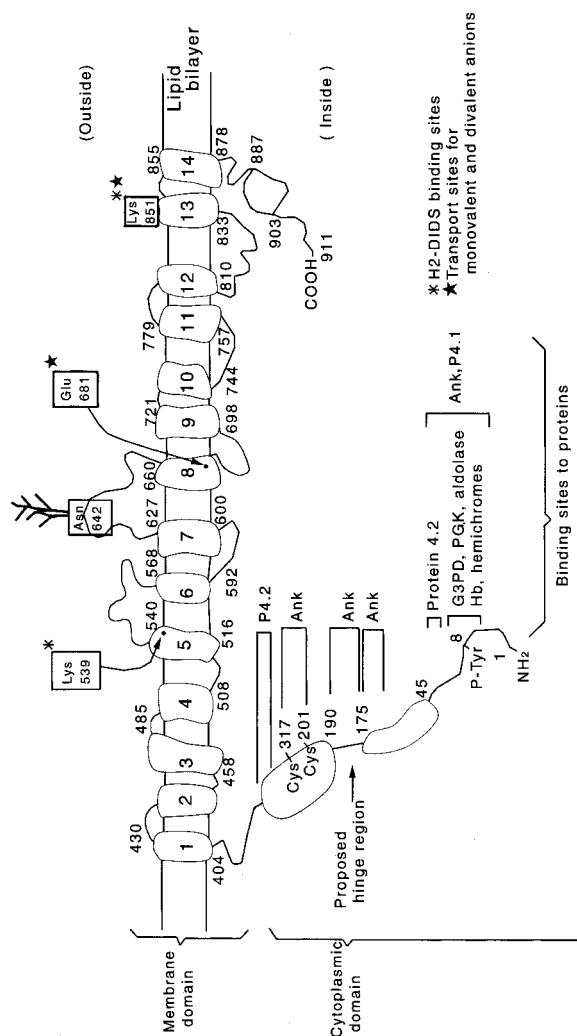


Figure 5.1 Schematic model for the molecular structure of human erythroid band 3. Band 3 molecules are composed of their cytoplasmic and transmembrane domains. The cytoplasmic domain demonstrates many binding sites to membrane proteins and cytoplasmic proteins. The membrane domain contains anion transport sites, such as *lys539*, *Glu681*, and *Lys851*. *Asn642* in the band 3 molecule is the binding site for a sugar chain.

in red cells of various species [7, 9]. The protein at position 403 is particularly important for creating a β bend or a random coil at the membrane junction, which is known as an inter-domain hinge and gives the tail freedom of movement [11].

5.1.2

Functions of Band 3

5.1.2.1 Membrane Protein Binding by the Cytoplasmic Domain of Band 3

The cytoplasmic (43 kDa) domain of band 3 plays a central role in the attachment of the cytoskeleton to the plasma membrane. The inter-domain hinge at this attachment point is important for the flexibility and rigidity of red cells. Three peripheral membrane proteins (ankyrin [12–15], protein 4.1 [16, 17], and protein 4.2 [18]) bind to the cytoplasmic domain of band 3. The ankyrin-binding involves several regions (proximal, middle, and distal) scattered throughout the cytoplasmic domain, implying that the cytoplasmic domain has a complex folded structure. Ankyrin binds to the flexed conformations of band 3, most probably to band 3 tetramers. It has been suggested that protein 4.1 and protein 4.2 have more than one site of attachment. Protein 4.1 also binds to peptides containing clustered basic residues (LRRRY and IRRRY) that are located at the COOH-terminal end of this domain [17]. Some of the band 3 gene mutations (band 3 Tuscaloosa, band 3 Montefiore, band 3 Fukuoka, and band 3 Coimbra; see Section 15.1) are known to demonstrate a markedly decreased protein 4.2 content.

Band 3 is associated with glycophorin A (GPA) in the membrane [19, 20]. GPA facilitates translocation of band 3 from the endoplasmic reticulum as the site of synthesis of band 3 to the plasma membrane of *Xenopus* oocytes. Band 3 is critical for GPA synthesis or its stability, since GPA is also deficient in the band 3-deficient red cells [21]. Glycophorins do not appear to be a determinant for the expression of band 3, because when there is a combined deficiency of glycophorin A and B (a glycophorin M^kM^k phenotype) the red cells demonstrate normal amounts of band 3. There is additional evidence for the interaction of band 3 with GPA. The Wright (W_r^b) antigen is caused by interaction of a site on band 3 (Glu⁶⁵⁸) with a site or sites located near the end of the extracellular domain of GPA or in the adjacent transmembrane domain [22]. A monoclonal antibody to the W_r^b blood type antigen immunoprecipitates both band 3 and GPA [23]. Furthermore, antibodies to glycophorin decrease the lateral and rotational mobility of band 3, indicating the presence of an interaction of band 3 with glycophorin.

Self-association of band 3 is one of its major functions in red cell biology. Band 3, which is extracted from the membrane by a nonionic detergent (octaethylene glycol n-dodecyl monoether), exists as stable dimers (70%), tetramers, and higher-order oligomers (30%) [24]. Tetramers of band 3 are associated with the membrane skeleton. Since isolated membrane domains only form dimers, tetramers appear to be assembled by cross-linking neighboring dimers through the cytoplasmic domain, with ankyrin, or hemichromes. The most efficient physiological functional form in which interactions with cytoplasmic molecules occur appears to be tetrameric.

5.1.2.2 Binding to Glycolytic Enzymes by the Cytoplasmic Domain of Band 3

The acidic NH₂-terminal sequence has the binding sites for the glycolytic enzymes, that is, glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) [25], phosphoglycerate kinase (PGK) [26], and aldolase [27]. These enzymes are basically cytosolic enzymes, but about 65 % of G-3-PD, 50 % of PGK, and 40 % of aldolase are bound to band 3 in the intact red cells. The enzymatic activities of these three enzymes are inhibited by membrane attachment, which are regulated by substrates, cofactors, inhibitors, and also phosphorylation of tyrosine 8 [28]. The phosphorus is added by the kinase p72syk and may be removed by a phosphotyrosine phosphatase which is bound to band 3.

5.1.2.3 Binding to Hemoglobin by the Cytoplasmic Domain of Band 3

The cytoplasmic domain of band 3 also binds hemoglobin. Deoxyhemoglobin binds better than the oxyhemoglobin. As regards this binding, the first five to seven amino acids of band 3 at the NH₂-terminal end are inserted deep into the 2,3-diphosphoglycerate (2,3-DPG)-binding cleft of hemoglobin [29]. Therefore, 2,3-DPG inhibits deoxyhemoglobin binding by band 3. Under physiological conditions, approximately half the band 3 molecules have hemoglobin attached. A denatured form of hemoglobin binds better and polymerizes with band 3, forming an aggregate of hemichromes and band 3 [30]. This binding of band 3 with hemichromes appears to stimulate aggregation into patches, which are uniquely recognized by a red cell senescence isoantibody, leading to opsonization of the cell and its removal from circulation by the spleen. This mechanism may be one of the important events in red cell ageing.

5.1.2.4 Anion Exchange Channel by the Transmembrane Domain of Band 3

The transmembrane (52 kDa) domain of band 3 (amino acids 404–911) contains an anion exchange channel [1, 31–34]. The transport capacity is evaluated as being from 10¹⁰ to 10¹¹ bicarbonate and chloride anions per second by 1200 × 10³ molecules of band 3 per red cell. The bicarbonate is produced by carbonic anhydrase in the red cells. Most of the bicarbonate produced appears to have originated through this channel. In addition, approximately 60 % of CO₂ transport from the tissues to the lungs appears to be handled in this way. The hydrogen (H⁺) byproduct by carbonic anhydrase binds to hemoglobin and facilitates oxygen release to the tissues [33]. All of these reactions are reversed in the lungs. The basic structural unit in both the inward- and outward-facing transporters is an oblong band 3 dimer. At the center of the dimer, the two monomers are located close together to form a channel. There is also a flexible subdomain on the far side of each monomer, which might be formed by one or more of the large cytoplasmic loops between transmembrane helices. The anion exchange itself appears to occur through a ping-pong mechanism. An intracellular anion enters the transport channel and is translocated outward and released, with the channel remaining in the outward conformation until an extracellular anion enters and

triggers the reverse cycle [1]. It has recently been shown that glutamic acid (Glu⁶⁸¹) plays the key role in chloride translocation by human band 3, that Glu⁶⁸¹ is located near the carboxy terminus of transmembrane segment 8 (TM8) at the region of the chloride channel, and that Lys⁸⁵¹, Ser⁸⁵², and Ala⁸⁵⁸ are also required for this channel [34].

Anion transport requires a high energy and volume of activation. Anion exchange is extremely rapid with a half-life of 50 ms for chloride and bicarbonate, and the specificity of the channel is fairly broad. At much slower rates, large anions (sulfate, phosphate, phosphoenol pyruvate, and superoxide) are also transported through this channel [1, 2].

5.1.2.5 Lateral and Rotational Mobility of Band 3

Lateral mobility of band 3 in normal human red cell membranes is constrained by steric hindrance interactions, low affinity binding interactions, and high-affinity binding interactions [35–37]. Steric hindrance interactions between band 3 oligomers and the spectrin-based membrane skeleton put a major constraint on the laterally mobile band 3 fraction, slowing the rate of band 3 lateral diffusion by approximately 50-fold compared with the predicted diffusion rate of free band 3 in membranes devoid of a functional membrane skeleton. The spectrin/band 3 ratio is the major determinant of the lateral diffusion rate of band 3. Fluorescence recovery after use of the photobleaching (FRAP) method (Fig. 5.2) demonstrates that, in normal red cells, approximately one-third of band 3 is present in the mobile fraction and the remaining two-thirds exist in the immobile fraction, which is fixed to the cytoskeletal network, in particular to ankyrin [35–37]. It has been reported that the mobile fraction was 0.43 ± 0.11 with a lateral diffusion coefficient of $(6.86 \pm 1.37) \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$ in normal red cells by the FRAP method.

Rotational mobility of band 3 in normal human red cell membranes is constrained by low-affinity and high-affinity binding interactions. The rotationally immobile band 3 fraction apparently represents individual band 3 molecules bound with high affinity to ankyrin. The rapidly rotating band 3 fraction consists of dimers, tetramers, and higher order oligomers of band 3 that are free from rotational constraints other than the viscosity of the lipid bilayer. The slowly rotating band 3 fraction is less well-defined. Rotational constraints applied by low-affinity binding interactions between ankyrin-linked band 3 and other band 3 molecules, and between the cytoplasmic domain of band 3 and membrane skeletal proteins (ankyrin, protein 4.1 and protein 4.2) have been invoked.

5.1.2.6 Blood Type Antigens and Band 3

On the transmembrane domain of band 3, a complex carbohydrate structure is attached to Asn⁶⁴² [1, 2, 20]. Within the carbohydrate structure, N-acetylglucosamine, mannose, galactose and other moieties exist.

Band 3 carries various polymorphic peptide epitopes including band 3 Memphis (Lys → Glu at position 56), such as the Diego blood group system, Wright (Wr^a and

(I) Procedure

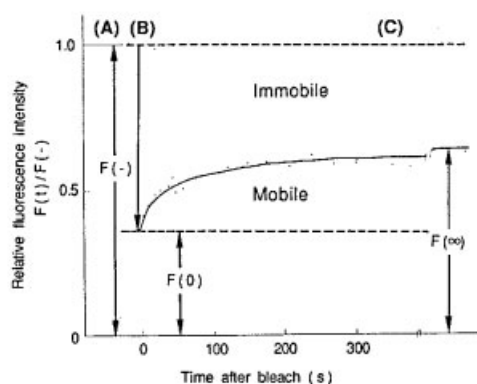
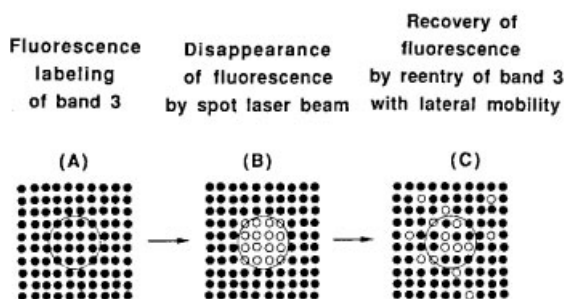
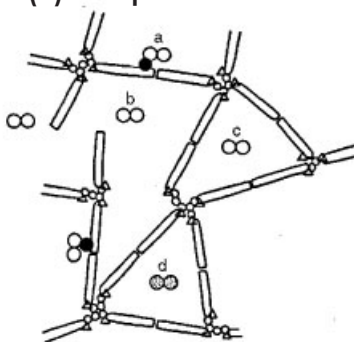


Figure 5.2 Principle of fluorescence recovery after the photobleaching (FRAP) method to examine lateral mobility of band 3 molecules in red cell membranes *in situ*. The procedure is shown in (I). After band 3 is labeled by fluorescence (A), it is subjected to a spot laser beam, which abolishes the intensity of fluorescence on band 3 (B). The intensity of fluorescence is recovered by reentry of band 3 molecules with their lateral mobility (C). Relative fluorescence intensity is measured during this

(II) Interpretation



- a. Immobile $D: <10^{-13} \text{ cm}^2/\text{s}$
- b. mobile $D: 5 \times 10^{-12} \sim 5 \times 10^{-11} \text{ cm}^2/\text{s}$
- c. mobile $D: 5 \times 10^{-12} \sim 5 \times 10^{-11} \text{ cm}^2/\text{s}$
- d. mobile $D: 4 \times 10^{-13} \text{ cm}^2/\text{s}$

- band 3
- ⊗ trypsinized band 3
- ankyrin
- spectrin hetero-dimer
- △ protein 4.1
- actin

course of time. The extent of the recovery of fluorescence intensity represents a mobile fraction (approximately 30 % of total) of band 3, and the remainder is equivalent to an immobile fraction of band 3. Interpretation of the results is shown schematically in (II). The band 3 molecules attached to the cytoskeletal network with anchoring proteins (ankyrin, and also probably protein 4.2) are immobile (a), and those without any fixation to the network are mobile (b, c, and d).

Wr^b) antigens and several other low frequency antigens [38]. No apparent consequence for band 3 function is observed in these antigenic variants.

The Diego (Di^b) allele is observed with varying frequencies in Asian and South American populations, although this allele is exceedingly rare in most Caucasians [20]. Di^a and Di^b antigens correspond to a proline or a leucine residue at position 854 of the band 3 molecule, respectively.

Among the Wright alleles, the Wr^b allele is predominantly expressed (higher than 99 %), whereas the Wr^a allele is extremely rare [20, 22, 23]. Wr^a and Wr^b anti-

gens correspond to a lysine or a glutamine residue, respectively, encoded by codon 658 of band 3. The expression of Wr^b is suppressed in glycophorin A deficiency.

Band 3 also carries several minor antigens, such as Waldner (Wd^a), Redelberger (Rb^a), Traversu (Tr^a), Wulfsberg (Wu), Moen (Mo^a), ELO, and Warrior (WARR). It is known that WARR and Wu correspond to point mutations of Thr 552 $\rightarrow$ Ile and Gly 565 $\rightarrow$ Ala of band 3, respectively. ELO, Rb (a+), Tr (a+), and Wa (a+) correspond to Arg 432 $\rightarrow$ Trp, Pro 548 $\rightarrow$ Leu, Lys 551 $\rightarrow$ Asn, and Val 557 $\rightarrow$ Met substitutions, respectively [20, 38].

On the transmembrane domain of band 3, a complex carbohydrate structure is attached to Asn⁶⁴². The Ii antigens are carbohydrate molecules and correspond to portions of the oligosaccharide chains [20]. Molecules with i reactivity correspond to oligosaccharide chains containing at least two repeating N-acetylgalactosamine units, whereas I activity corresponds to a branched oligosaccharide structure formed by an N-acetylgalactosamine unit attached in a β 1,6-linkage to a galactose residue within linear lactosamine polymers. Oligosaccharide chains in neonatal red cells are largely unbranched, and those in adult red cells are highly branched. The increase in the I reactivity, with a corresponding decrease in i reactivity, during early infancy corresponds to the elaboration and display of increasing numbers of β 1,6-linked lactosamine units. Therefore, red cell expression of these Ii antigens is developmentally regulated. This particular β (1,6) N-acetylglucosaminyltransferase activity and a chain-elongating β (1,3) N-acetylglucosaminyltransferase activity have been identified.

5.1.3

Band 3 in Nonerythroid Cells

It is known that, in addition to erythroid band 3 (anion exchanger-1: AE1, or solute carrier family 4A: SLC4A1), two other genes (AE2 and AE3) encoding band 3-related anion exchange proteins are present [7, 8, 39–41]. AE2 (or SLC4A2) is the general tissue anion antiporter, and is widely distributed in many tissues and cells. AE3 (or SLC4A3) is expressed in the heart and brain. In AE2 and AE3, approximately 300 amino acids are added to the NH_2 -terminus of the AE1 molecule. Therefore, AE2 and AE3 are larger than AE1. Among these three transporters, there is distinct homology, particularly in the membrane domain of their molecules. AE1 is also expressed in tissues other than red cells, such as in the acid-secreting, type A-intercalated cells in the collecting ducts of the kidney, and in cardiac myocytes. The kidney transcript lacks the first 66 amino acids of the cytoplasmic domain of AE1. Thus, it is unable to bind glycolytic enzymes, protein 4.1, or ankyrin.

5.2

Glycophorins

The glycophorins are known as the most abundant integral membrane glycoproteins in red cells [19, 42, 43]. Glycophorins have a high sialic acid content, and

more than 95 % of the periodic acid Schiff (PAS)-staining compounds come from the glycophorins. The five types of glycophorins are known as glycophorins A, B, C, D, and E [19, 42–52]. Among these five glycophorins, glycophorins A, B, and E are encoded by three closely linked genes [49–52], whereas glycophorins C and D arise from a single locus bearing no significant homology to the genes for glycophorins A, and B. Glycophorin D differs from glycophorin C by use of an alternate translation start site created by alternative mRNA splicing [19, 42, 43]. Another gene linked in tandem with those for glycophorins A and B has been isolated, which encodes glycophorin E [49–52]. This appears to have evolved from glycophorin A by homologous recombination at *Alu* repeats [49, 50]. However, no protein product has been identified regarding the gene for glycophorin E. Characterization of cDNA and genomic clones encoding the glycophorins has revealed that they fall into two distinct subgroups. Therefore, glycophorins are categorized into two groups, that is: (1) glycophorins A, B, and E, and (2) glycophorins C and D [19, 42, 43].

5.2.1

Glycophorins A, B, and E

5.2.1.1 Glycophorin A (GPA)

Glycophorin A (GPA) is the major sialoglycoprotein of red cells [19]. It is present at a level of approximately one million copies per cell (Tables 1.1 and 1.2). Its molecular weight, including carbohydrate, which is estimated from mobilities on SDS gels is 36×10^3 , and the molecular weight which is calculated from its molecular sequence excluding the carbohydrate chain is 14×10^3 . GPA is approximately 1.6 % (w/w) of the total membrane proteins in the red cells. Three GPA complementary DNA transcripts are expressed from the gene (transcripts of 2.8, 1.7, and 1.0 kb) that vary only by the use of alternative polyadenylation sites. The 5'-ends of these transcripts are essentially identical.

The gene of GPA (*GYPA*) is located at chromosome 4q28–q31. GPA is synthesized with a cleavable leader peptide that yields a type I transmembrane protein 131 amino acids long [19, 44, 45]. The protein is heavily glycosylated with a single asparagine (position 26)-linked glycan and 15 serine–threonine-linked oligosaccharide units. Approximately 60 % of the GPA molecule is carbohydrate.

GPA gene is composed of seven exons (Fig. 5.3). Exon 1 yields a leader peptide, whereas exons 2 (amino acids 1–26), 3 (amino acids 27–57), and 4 (amino acids 58–70) encode the extracellular domain. Exon 5 encodes the transmembrane domain (amino acids 71–100). Exons 6 and 7 generate the cytosolic domain (amino acids 101–131) and 3'-untranslated region [19, 44, 45].

The precise biological functions of GPA have not been well defined. Since GPA demonstrates an extensive negative surface charge of the red cells, it is expected that it may modulate interactions between red cells and red cells, or between red cells and endothelial cells. It has been reported that red cells are clumped when sialic acids are removed. The M and N antigens on GPA are determined by amino acid polymorphism at positions 1 and 5 of the mature polypeptide. The M antigen is defined by a serine at amino acid position 1 and a glycine at position

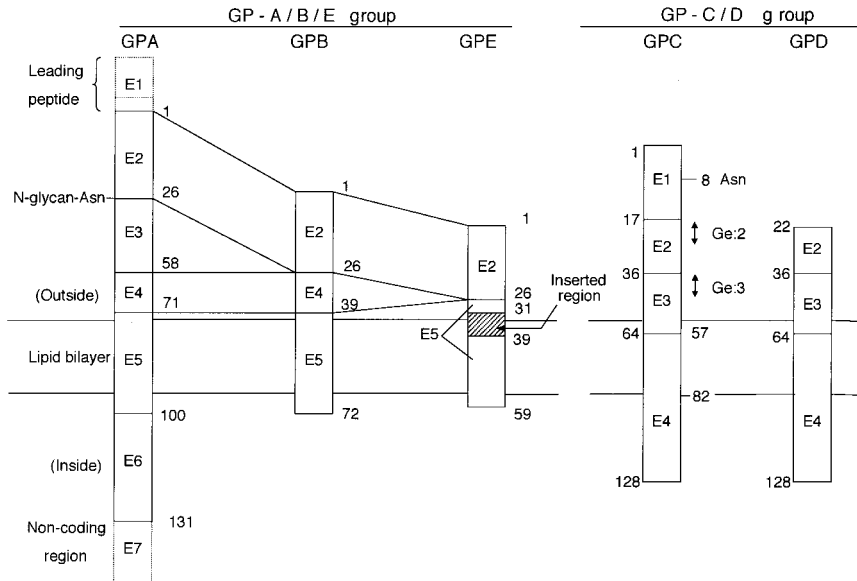


Figure 5.3 Molecular structures of glycoproteins A, B, C, D, and E. Glycoproteins B (GPB) and E (GPE) are basically derived from glycoprotein A (GPA). In contrast, glycoprotein D (GPD) is derived from glycoprotein C (GPC). E denotes exons. Ge: Gerwich blood group antigens.

5, whereas the N antigen is defined by a leucine at position 1 and a glutamine at position 5 [19, 20, 53]. Another example is the Ss phenotype. When methionine is present at amino acid position 29, the S phenotype is expressed [19, 20, 53]. Similarly, the presence of threonine at position 29 produces the s phenotype.

GPA is expressed only in erythroid cells, especially after the proerythroblast stage during terminal erythroid maturation. Glycophorin A-deficient red cells, such as an En (a-) type [20, 54], exhibit increased glycosylation of band 3, probably due to the addition of excessive sialic acid, which should have been present on the GPA molecule. Therefore, total surface charge density is not affected, and GPA-deficient red cells maintain the normal red cell shape and deformability. Thus, GPA may not be crucial with respect to the maintenance of mechanical stability, deformability, or shape change. Total deficiency of band 3 in the band 3-knock-out (-/-) mice [55, 56] or in the Japanese cow [57] is associated with complete deficiency of GPA in their red cells. In addition, when red cells bind to immunologically non-specific ligands, (wheat germ agglutinin) the binding causes aggregation of glycophorin and decreases red cell deformability. Glycophorin-deficient red cells are more resistant to invasion by malaria parasites than are normal red cells [58]. Amino acids 90 to 93 of the membrane domain appear to be critical to the formation of GPA dimers in the membranes. In the transmembrane domain, a GPA dimer showed a small, well-packed interface between the molecules. Van der Waals interactions alone can mediate stable and specific associations between transmembrane helices [59].

5.2.1.2 Glycophorin B (GPB)

Glycophorin B (GPB) is fairly similar to GPA except for their exoplasmic domains and cytoplasmic tails [46, 48]. The gene for GPB (*GYPB*) is located on chromosome 4q28–q31 (Tables 1.1 and 1.2). GPB is a structurally similar type I transmembrane protein derived from a gene consisting of five exons. This gene yields a single 0.5 kb transcript. GPB is synthesized with a cleavable single sequence to yield a type I transmembrane protein, which is 72 amino acids in length. There are no asparagine-linked carbohydrate chains, because asparagine in position 26 of exon 3 is spliced out, whereas there are approximately 11 serine–threonine-linked oligosaccharide chains. Approximately 50 % of the mass of GPB molecules consists of oligosaccharide. There are only about 150 000 copies per cell. The apparent molecular weight of GPB on the SDS gels is 20×10^3 , and that calculated from protein sequence data is 8×10^3 . GPB is equivalent to 0.2 % (w/w) of the total red cell membrane proteins.

The glycophorin B gene is composed of five functional exons (exons 1, 2, 4, 5, and 6) [46, 48] (Fig. 5.3). Sequences corresponding to exon 3 are designated as pseudoexons, because the sequences are not expressed in GPB transcripts as a consequence of a non-functional splice acceptor sequence at its 3'-border. Exon 1 yields a leading peptide. The extracellular domain is encoded by exons 2 and 4, and exon 5 encodes the transmembrane and short cytosolic segment. Exon 6 generates the 3'-untranslated region. Amino acid sequence polymorphisms encoded by exon 4 yield an S-specific GPB molecule (methionine at position 29), or an s-specific molecule (threonine at position 29) [20, 53]. The amino acid sequence encoded by exon 2 yields the N antigen, with leucine at position 1 and glutamine at position 5.

No biological function has been assigned to GPB other than its association with the Ss blood group [20, 53]. GPB is only present in erythroid cells, as is GPA.

5.2.1.3 Glycophorin E (GPE)

Glycophorin E is a glycophorin that has been identified by molecular cloning [49–52] (Tables 1.1 and 1.2). The genes for glycophorins A, B, and E are located on the same chromosome 4q28–q31, in the order A, B, and E. The genomic structure and promoters of all three genes are highly conserved, and all three contain cleavable leader peptides. These three genes are oriented in a tandem fashion in a gene cluster (Fig. 5.4).

The glycophorin E gene (*GYPE*) is predicted to be composed of four functional exons (exons 1, 2, 5, and 6) and two non-utilized pseudoexons (numbers 3, and 4) (Fig. 5.3). Exon 1 yields a leader peptide, exon 2 (positions 1 to 26) encodes the putative extracellular domain, and exon 5 (positions 26 to 59) encodes the predicted transmembrane segment. Exon 6 produces the 3'-untranslated region. The *GYPE* locus lacks DNA sequences corresponding to amino acid residues 27 to 39 of GPB, encompassing the position of the Ss amino acid sequence polymorphism in GPB. *GYPE* also contains a DNA sequence insertion, relative to *GYPB* and *GYPE*, at a position corresponding to exon 5 of *GYPB*. This insertion is predicted to encode eight amino acids not present in GPA or GPB. A polypeptide product corresponding

to the *GYPE* locus has not been identified *in vivo*, but it could be a 20 000 Da molecule with a length of 59 amino acids, and with residues 1 and 5 occupied by serine and glycine, respectively, corresponding to the M type blood group.

5.2.2

Glycophorins C and D

5.2.2.1 Glycophorin C (GPC)

Glycophorin C (GPC) is a glycoprotein with 128 amino acids [42, 43, 47, 48, 60, 61]. The molecular weight on the SDS gels is 32 kDa, but when calculated it is 14 kDa, because extensive posttranslational modification by glycosylation at the single asparagine (residue 8)-linked N-glycosylation site and 12 serine–threonine-linked O-glycosylation sites are present (Tables 1.1 and 1.2). Approximately 0.1 % of the total membrane proteins (w/w) is GPC. There are about 143×10^3 copies of glycophorins C (per red cell). The gene for GPC (*GYPC*) is located on chromosome 2q14–q21. The GPC gene is composed of four exons (Fig. 5.3). The extracellular domain of GPC (residues 1–57) is encoded by exons 1, 2, and 3. The glycosylation sites are located in this extracellular domain. The transmembrane segment (residues 58–81) is encoded by exons 3 and 4, and the cytosolic domain (residues 82–128) by exon 4. The Gerbich (Ge: 2 and Ge: 3) blood type antigens are located at positions corresponding to exons 2 and 3 of GPC, respectively [20, 42, 62].

It is known that GPC is not erythroid-specific, and is present in multiple nonerythroid tissues in a distinctive fibrillar pattern. During erythroid differentiation, a desialylated form of GPC is present on the surface of erythroid progenitors of the burst forming unit in the erythroid (BFU-E). Normally glycosylated GPC first appears in erythroid progenitors of the colony-forming unit in erythroid (CFU-E).

GPC is functionally important, because the cytoplasmic tail of GPC binds to protein 4.1 and p55 resulting in the anchoring of the skeletal network to the membrane, as described previously (in Sections 4.2.2 and 4.4.1). Therefore, GPC plays a crucial role in regulating the stability, deformability, and shape of the membrane.

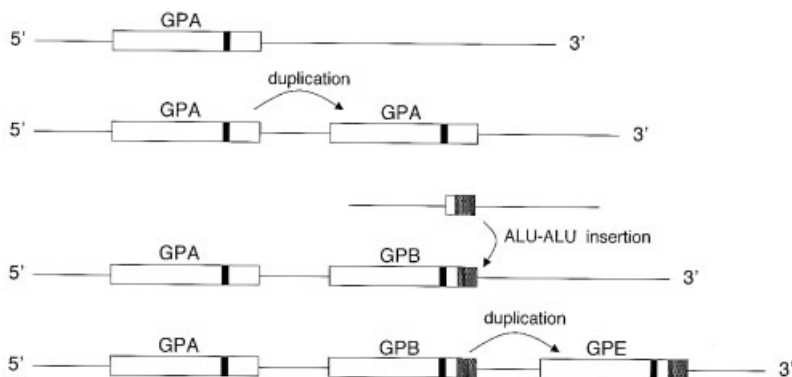


Figure 5.4 Evolution of glycophorins A, B and E. GPA: glycophorin A, GPB: glycophorin B, and GPE: glycophorin E.

5.2.2.2 Glycophorin D (GPD)

GPD is a truncated form of GPC (Fig. 5.3), corresponding to residues 22 to 128 of GPC [20, 42, 43, 60]. Therefore, GPD (23 kDa) is approximately 9000 Da smaller than GPC (32 kDa) on the SDS gels (Tables 1.1 and 1.2). The molecular weight of GPD calculated from the gene (*GYPD*) sequence is 11 kDa, and roughly 0.02% of the total membrane proteins (w/w) is GPD. There are approximately 82×10^3 copies of GPD per red cell. The same transcript yields both GPC and GPD through a mechanism involving translation initiation at an internal ATG codon. When translation is initiated at the first AUG, GPC is produced. When initiation occurs at the AUG encoding methionine at position 20, GPD as a truncated protein is produced. Residues 22 to 128 are identical in GPC and GPD. GPC does not express a cleavable signal peptide. The genes for GPC and GPD are located on chromosome 2q14–q21. There are six O-linked sugar chains in the GPD molecule, but none of the N-linked sugar chains. GPD expresses only the Ge: 3 determinant of the Gerbich blood group [20, 62].

5.3

Blood Group Antigens

As many as 243 different determinants have been listed as blood group antigens, which belong to one of 19 distinct blood group systems [20, 63–68] (Table 5.1). Some of these demonstrate the clinical relevance of the antigens especially in red cell transfusion and organ transplant procedures. Therefore, current information on their biochemical and genetic properties and the molecular basis for the inherited polymorphisms in these molecules is critical.

5.3.1

ABO Blood Group

Human beings are classified into distinct groups by the ABO blood group system (Table 5.1) depending on the presence or absence of substances in the serum that agglutinate red cells from humans of other classes [69]. The antigens of the ABO system (A, B, and H determinants) are expressed by red cells and by many other tissues. The cells of some human tissues produce water-soluble forms of these molecules as components of the glycans on secreted and soluble glycoproteins, on glycosphingolipids, and on free oligosaccharides. This mechanism is determined by the *Secretor (Se)* locus.

The immunoreactive regions of the ABO blood group determinant are located at the terminal ends of various oligosaccharides, which are components of integral proteins and glycolipids of red cell membranes [69–72]. The A, B, and H blood group molecules are constructed sequentially by distinct glycosyltransferases which are determined by a distinct genetic locus. These glycosyltransferases operate on one of four structurally distinct oligosaccharide precursor types synthesized in human cells. Type 1 oligosaccharide precursors are found at the termini of lin-

Table 5.1 Major human blood group systems.

ISBT No.	System name/symbol	Gene name	Antigen type	Antigen copy number per red cell	Number of alleles	Chromosome	Chapter No. in text
001	ABO/ABO	ABO	Oligosaccharide	$8 \times 10^5 - 2 \times 10^6$	3 major	9q34.1–q34.2	5.3.1
002	MNS/MNS	GYPA, (GYPE)	Glycoproteins	$\sim 1 \times 10^6$	Several minor (cis-AB, subgroups)	4q28–q31	5.2.1
	MN (glycophorin A)				2 major (M or N)		
003	S (glycophorin B)	GYPB		$\sim 1.5 \times 10^5$	Multiple minor	4q28–q31	5.2.1
	P/P1		Oligosaccharide	$\sim 10^3$	2 major (S or s)		
004	Rh/RH	P1	Protein complex		Multiple minor	22q11.2–qter	5.3.3
	D				Complex		
005	C/c, E/e	RHCE		$\sim 2 \times 10^4$	~ 48 haplotypes	1p34.3–p36.1	5.3.2
	Lutheran/LU			$\sim 2 \times 10^4$	Includes minor C/c and E/e alleles		
006	Kell/KEL	KEL	Glycoprotein	$\sim 1600-4100$	2 major (D ⁺ or D ⁻)	7q33	5.3.4
	Lewis/LE		Glycoprotein	$\sim 5 \times 10^3$	2 major (C or c, and E or e)		
007	Duffy/FY	FUT3		4500–7300	3 major (Lu ^a , Lu ^b , recessive null)	19q12–q13	5.3.5
	Kidd/JKkidd/JK ^a ,4s			$\sim 12\,000-17\,000$	2 major (KEL1, KEL2)		
008	Diego/DI	AE1		$\sim 15\,000$	Several minor	19p13.3	5.3.6
					2 major (Le ^a , Le ^b)		
009		FY		$\sim 14\,000$	2 major (Fy ^a and Fy ^b)	1q22–q23	5.3.7
					2 major (Jk ^a , Jk ^b)		
010		ACHE			Several	18q11–q12	5.3.8
					Di ^a , Di ^b		
011	Cartwright/YT	ACHE			Wt ^a , Wt ^b	17q12–q21	5.3.11
					Other high-frequency antigens		
012	XG/XG	XG	Glycoprotein	Not determined	1 major (Yt ^a)	7q22	5.3.9
			(acetylcholinesterase)		1 minor (Yt ^b)		
013		XG			1 major (Xg ^a)	Xp22.32	5.3.10
					1 minor (Xg, a hypothetical null)		

Table 5.1 Continued.

ISBT No.	System name/symbol	Gene name	Antigen type	Antigen copy number per red cell	Number of alleles	Chromosome	Chapter No. in text
013	Scianna/SC	SC	Glycoprotein	Not determined	3; Sc1, Sc2, and Sc3	1p36.2–p22.2	
014	Dombrock/DO	DO	Glycoprotein (GPI-linked)	Not determined	1 major (Do ^a) 1 minor (Do ^b)	unknown	
015	Colton/CO	AQP1	Glycoprotein (aquaporin-1)	Not determined	2 major (Co ^a and Co ^b)	7p14	
016	Landsteiner-Weiner/LW	LW	Glycoprotein (ICAM-4)	~4400 (on D ⁺ cells) ~2835–3620 (on D ⁻ cells)	2 major (LW ^a , LW ^b) Rare nulls	19p13.3	5.3.9
017	Chido-Rogers/CH/RG	C4A, C4B	Glycoprotein (4th component of complement; C4A and C4B)	~10 ³	Ch (1 major, several minor) Rg (1 major, several minor)	6p21.3	
018	Hh/H	FUT1	Oligosaccharide	See ABO	1 major Several minor	19q13	
019	Kx/XK	XK	Protein	Not determined	(Bombay, para-Bombay) 1 major (K ^x)	Xp21.1	
020	Gerbich/GE	GYPE, GYPD	Glycoproteins	~1 × 10 ⁵ (glycophorin C) ~2 × 10 ⁴ (glycophorin D)	1 major (Ge: 1, 2, 3, 4) Multiple minor	2q14–q21	5.2.2
021	(glycophorins C and D) Cromer/CROM	DAF	Glycoprotein (decay-accelerating factor)	~10 ³	2 major (Cr ^a , Cr ^b) Several minor	1q32	
022	Knops/KN	CR1	Glycoprotein (complement receptor CR1)	Not determined	Several major (Kn ^a , Kn ^b , Mc ^a , Mc ^b , Yk ^a , SI ^a)	1q32	
023	Indian/IN	CD44	Glycoprotein	Not determined	2 major (In ^a , In ^b)	11p13	
–	Secretor/Se	FUT2	Oligosaccharide	see Lewis	2 major (Secretor, non-Secretor)	19q13	
–	Ii		Oligosaccharide	see ABO	1 major (I), at least 1 minor (i)	9q21	5.3.10

ISBT: International Society of Blood Transfusion.

ear and branched chain oligosaccharides linked to proteins at asparagine residues or at serine or threonine residues.

The type 1 oligosaccharide precursors are synthesized only by epithelia of various tissue cells, yielding ABH determinants present in body fluids and secretions. The ABH determinants expressed by red cells are mainly by the type 2 precursor chains, which are also asparagine-linked or serine–threonine-linked oligosaccharides. Type 3 A, B, and H antigens in mucins are not found in human red cells. Type 4 chains are restricted to glycolipids in human red cells.

These oligosaccharide precursors are catalyzed by α (1,2) fucosyltransferase in a transglycosylation reaction. These enzymes transfer the nucleotide sugar substrate GDP-fucose to carbon 2 of the galactose molecule at the oligosaccharide precursors [69, 70]. The fucose is attached in an alpha anomeric linkage and forms the blood group H determinant, such as the disaccharide Fuc α (1,2) Gal β -unit. The human genome encodes two different α (1,2) fucosyltransferases in a tissue-specific fashion, which correspond to the products of the *H* and the *Se* blood group loci [73].

Glycosyltransferases, which are encoded by the ABO blood group locus, use type 1, 2, 3, or 4 H determinants to form A or B blood group determinants. The A allele at the ABO locus encodes an α (1,3) N-acetylgalactosaminyltransferase that uses H molecules to form the blood group A molecule [69–71]. The B allele encodes an α (1, 3) galactosyltransferase that operates on H-active oligosaccharide precursors to form the blood group B determinant. The O allele is a null allele, which cannot encode a functional glycosyltransferase that will further modify H-active precursors.

The ABH determinants in human red cells are mainly associated with membrane glycoproteins, that is, 80% of those ($1-2 \times 10^6$ molecules per red cell) in band 3, and others in the red cell glucose transporter (band 4.5: 0.5×10^6 molecules per red cell), the Rh-related proteins, and the aquaporin-1 glycoprotein [74]. Red cell membrane glycolipids are also involved with the ABH molecules (0.5×10^6 molecules per red cell). A single asparagine-linked oligosaccharide molecule on band 3 is a branched poly-N-acetylgalactosaminoglycan, whose terminal branches may display several ABH determinants.

Ig M antibodies specific for ABO oligosaccharide determinants are not displayed in red cells during infancy. This immune response is a consequence of exposure to microbial oligosaccharide antigens that are structurally similar (or identical) to the A and B blood group molecules. It is also interesting to note that, in most individuals, antibodies directed against H determinants are not formed because a substantial number of the blood group H precursors are not enzymatically converted into A or B determinants. The Ig M isoagglutinins, which occur naturally, efficiently fix complement leading to acute hemolysis of transfused red cells that display the corresponding antigen. There are a substantial number of variants of the ABH blood group antigens [75, 76]. For example, in A subgroups, the A_1 and A_2 phenotypes are known, which differ in their molecular structure [70, 71]. The absolute number of immunodominant molecules is greater on A_1 cells than it is on A_2 cells.

The human A transferase is a type II transmembrane protein, and is composed of 353 amino acids with an NH₂-terminal segment (residues 1–15), a hydrophobic segment (residues 16–39), and a COOH-terminal domain (residues 40–353). The COOH-terminal catalytic domain is located within the membrane-delimited compartments of the Golgi and the trans-Golgi network, where terminal glycosylation reactions occur. This enzyme has a single potential site for asparagine-linked glycosylation, and functions as a glycosyltransferase [77].

The A transferase is also present as a soluble, catalytically potent polypeptide. In the molecular structure, the NH₂-terminus of the soluble enzyme corresponds to the alanine residue at codon 54, indicating that the soluble form of the A transferase is derived from its transmembrane precursor by proteolysis. Therefore, glycosyltransferase exists in both membrane-associated and soluble, catalytically active forms [70].

As regards the molecular basis for polymorphism at the *ABO* locus, the blood group O phenotype is produced and is due to a single base pair deletion of one nucleotide in the codon for amino acid 87 of the A transferase. By this frameshift of the reading frame, a termination codon appears at amino acid residue 117 of the A transferase. The truncated protein (116 amino acids) is consequently unable to modify the H antigen to form A or B structures, resulting in no detectable A or B transferase activity being observed in the sera taken from blood group O individuals (genotype OO).

As for the A or B blood group phenotype, seven differences in nucleotide sequence are present within the protein-coding segments of the A transferase cDNA. Three of the seven appear to be functionally neutral polymorphisms. The other four appear to be critical for expressing the A and B transferases. These are at residues 176 (arginine, A; glycine, B), 235 (glycine, A; serine, B), 266 (leucine, A; methionine, B), and 268 (glycine, A; alanine, B). The polymorphisms at positions 266 and 268 are important for enzymatic functions to discriminate between UDP-N-acetylgalactosamine and UDP-galactose. Therefore, leucine at 266 and glycine at 268, or methionine at 266 and alanine at 268 generate an A transferase or B transferase, respectively.

H blood group oligosaccharide precursors are the most important substrates for the transferases encoded by the *ABO* locus. H-active precursors display terminal Fuc α (1,2) Gal β linkages, which are an integral part of the A and B antigenic determinants. These linkages are synthesized by α (1,2) fucosyltransferases (GDP-fucose: Gal β 2- α -L-fucosyltransferase). These transferases can use types 1, 2, 3, and 4 glycoprotein or glycolipid substrates as well as low molecular weight β -D-galactosides [70].

The synthesis of H-active blood group substances is determined by two factors, that is, the *H* locus and the *Secretor* (*Se*) locus [69, 70].

The *Se* locus determines expression of an α (1,2) fucosyltransferase activity, and H-active blood group substances (membrane-associated, and also soluble). Nearly all of this soluble blood group-active substance is constructed from type 1 precursors and is released mainly from the sublingual and submaxillary glands and the parotid gland.

Red cells taken from both secretors and non-secretors maintain an essentially identical complement of H-determinants and also A or B determinants, depending on the *ABO* locus genotype. In red cell precursors, the synthesis of α (1,2) fucosyltransferase activity, and H determinants is directed by the *H* locus. The *H* and *Se* loci correspond to distinct genes encoding different α (1,2) fucosyltransferases with disparate tissue-specific expression patterns. The *H* locus is expressed predominantly in erythroid cells. In contrast, the *Se* locus represents a second α (1,2) fucosyltransferase locus whose expression is restricted to the epithelia of many tissue cells. Individuals with the secretor phenotype maintain at least one functional allele at both the *H* locus and the *Se* locus. Individuals with the non-secretor phenotype maintain two null alleles at the *Se* locus and at least one functional *H* allele.

In human erythroid cells, the *Se* locus is constructed mainly from type 1 precursors, and the H determinants, which are synthesized by the *H*-encoded α (1,2) fucosyltransferase, are based on type 2 precursors [69, 73]. The human *Se* locus and the human *H* locus are separated only by 35 kb of genomic DNA on the same human chromosome 19.

The human *H* locus encodes a 365 amino acid-long polypeptide as the type II transmembrane glycosyltransferase [78, 79]. The molecular structure is composed of an NH₂-terminal cytosolic domain (residues 1–8), a hydrophobic domain (residues 9–25) that spans the Golgi membrane, and a COOH-terminal domain (residues 26–365) corresponding to a Golgi-localized catalytic domain, where two potential asparagine-linked glycosylation sites are present. Therefore, the human *H* locus is an α (1,2) fucosyltransferase.

The human *Se* locus is a 332 or 343 amino acid-long polypeptide, which shares 68 % of the amino acid sequence identity with the COOH-terminal 292 residues of the human H blood group α (1,2) fucosyltransferase [79]. The molecular structure is composed of a cytosolic domain with 3 or 14 residues, a 14 residue hydrophobic membrane domain, and a 315 amino acid-long COOH-terminal domain, which is located in the Golgi lumen. There are three potential asparagine-linked glycosylation sites [80, 81].

5.3.2

Rh Blood Group

The Rh (Rhesus) blood group antigens were initially identified by Landsteiner and Wiener in 1940 in the antisera of immunized guinea pigs and rabbits with red cells taken from *Macaca rhesus* monkeys [20, 68, 82, 83]. The human alloantibody is called Rh, and the heteroantibody is called LW (Table 5.1). RH and LW are antigenic systems determined by distinct gene complexes, which are located on chromosomes 1 and 19, respectively. LW and the Rh antigen complex associate in the membrane, and LW expression requires Rh polypeptide expression. A third antigen, Rh50, is also important for normal expression of the Rh, LW, and glycophorin molecules.

The incidence of Rh positive is approximately 85 % in whites, and strikingly 99.5 % in the Japanese population. About 15 % of Caucasians and 0.5 % of Japanese

are Rh negative. One main determinant of the Rh antigen system is the RhD antigen. Therefore, Rh positive individuals demonstrate the RhD antigen, whereas the Rh negative ones do not express the RhD antigen. In the Rh blood group system, other antigens, that is, the C/c and E/e antigen group, are known [84]. Their expression is determined by a second locus linked extremely tightly to the locus that determines D antigen expression [82, 83].

There are eight common Rh gene complexes considering *D*, *C*, *c*, *E*, and *e* alleles. The *d* antigen does not exist, because there is no product of the hypothetical *d* allele of the *D* gene.

The C/c and E/e antigens correspond to a single, non-glycosylated, 417 amino acid-long (33 100 Da) polypeptide (RhCE) with 12 membrane spanning domains, encoded by the *RHCE* gene. The *RHCE* gene is composed of 10 exons in 70 kb of genomic DNA.

The D antigen corresponds to a distinct non-glycosylated, 417 amino acid-long (33, 100 Da) protein (RhD), which is encoded by the *RHD* gene. This RhD protein shares 90 % of the identity of the amino acid sequence with the RhCE protein, and also demonstrates a 12 membrane spanning domain [85]. The *RHD* and the *RHCE* genes are closely linked on human chromosome 1p34–p36.

Considering the Rh antigen system, there is a third polypeptide (Rh50), which is a 409 amino acid-long glycoprotein. This peptide is approximately 36 % identical with amino acid sequences of RhD and RhCE, which are also known as the Rh30 polypeptides [86]. This Rh50 peptide has a 12 membrane-spanning domain, and is encoded by the *RH50* gene, which is located on human chromosome 6p11–p21.1. The *RH50* gene exhibits 10 exons and 32 kb of its size, closely resembling those of the *RHD* and *RHCE* genes [87, 88]. Although the Rh50 protein does not itself express Rh antigens, it interacts with the Rh30 polypeptides (RhD and RhCE) in the membrane, and is required to form a heterotetrameric complex, two molecules of Rh50 and one molecule each of RhD and RhCE, which is essential to normal cell surface expression of the Rh30 (RhD and RhCE)-encoded Rh antigens.

The RhD and RhCE polypeptides are modified by fatty acylation and are also palmitylated through a thiolester linkage to free sulfhydryl groups on cysteine residues within a consensus tripeptide (Cys-Leu-Pro) in the molecules of the RhD and RhCE. Reactivity of Rh antigens is also regulated by the lipid composition of the membrane *in situ*. Therefore, alteration of the membrane lipid concentration could induce conformational changes of the Rh peptides. It is also known that the Rh polypeptides interact with the red cell membrane skeleton.

It has recently been shown that protein 4.2-deficient red cells lack CD47 implicating an interaction between the Rh complex and the band 3 complex [89].

5.3.3

P Blood Group

The P antigens (ISBT No.003: P/PI) are present exclusively on red cell membrane-associated glycosphingolipids [20, 68, 90, 91]. P molecules are produced from lactosyl ceramide by the sequential processing of a series of distinct glycosyltrans-

ferases (Table 5.1). Two distinct pathways have been proposed for the biosynthesis of the P antigen molecule via the step of the P^K antigen as a precursor, even though so little is known about the corresponding enzymes and genes.

The most common phenotype (P_1) demonstrates full activity, and its frequency is approximately 75 %. The other most common phenotype is P_2 with a frequency 25 %. Three rare phenotypes have been described: (1) the P_1^k phenotype, which is deficient in P transferase activity, (2) the P_2^k phenotype, which is homozygous for null alleles at the P transferase and the P_1 transferase loci, and (3) the p phenotype, which is deficient in all three P antigens (P, P_1 , and P^k).

Functions of the P blood group system remain unknown.

5.3.4

Lutheran Blood Group

In the Lutheran blood group system (ISBT No.005: Lutheran/LU), four major phenotypes are present [20, 68]. Approximately 90 % of normal subjects demonstrates Lu (a–b+), or LU: –1, 2 (ISBT phenotype). Others are Lu (a+b+); LU: 1, 2, Lu (a+b–); LU: 1, –2, and Lu (a–b–); LU: O, which displays no detectable Lutheran antigenic activity (Table 5.1). In blood, the Lutheran blood group proteins are restricted in their expression to red cells and to B lymphocytes.

The Lutheran antigens are expressed on a pair of membrane glycoproteins with molecular weights of 78 000 and 83 000. The Lutheran blood group antigens are a 597 amino acid-long type 1 transmembrane protein. The extracellular domain contains five potential N-glycosylation sites and five peptide segments that share a primary sequence similarity with members of the immunoglobulin superfamily [92]. Its structural organization is similar to MUC18 (the melanoma-associated, mucin-like protein) and related neural cell adhesion molecules. A single membrane-spanning segment is followed by a 59 amino acid-long intracellular segment, in which an Src homology 3 domain is present.

The Lutheran blood group gene (*LU*) yields a pair of alternately spliced transcripts, producing two molecular weight isoforms of the protein that differ in their lengths of the cytosolic domains. One isoform of the Lutheran polypeptide is identical to a B-CAM (a basal cell carcinoma/epithelial cancer adhesion molecule). The Lutheran blood group gene corresponds to a 12.5 kb gene with 15 exons [93, 94]. There are several nucleotide polymorphisms in exon 3 at base pair 229 of the coding sequence. In the Lutheran (a+b–) phenotype, the nucleotide A at this position contributes to a histidine codon corresponding to residue 77 of the Lutheran polypeptide. In the (a–b+) phenotype, the nucleotide at this position is G corresponding to an arginine codon at residue 77 of the peptide.

5.3.5

Kell Blood Group

Although many human blood group alloantigens have been discovered, the KEL1 antigen of the Kell blood group system (ISBT No.006: Kell/KEL) is highly immu-

nogenic, behind the RhD antigen, which is the strongest in its immunogenicity [20, 68, 95, 96]. Anti-KEL1 antibodies account for approximately two-thirds of non-Rh immune red cell alloantibodies. There are approximately 5000 Kell determinants per red cell (Table 5.1).

The Kell protein is a 732 amino acid-long 83 000 Da polypeptide. This is a type 1 transmembrane protein with a cytosolic NH₂-terminal segment, a single hydrophobic membrane-spanning segment, and a large COOH-terminal extracellular domain [95, 96]. Six potential asparagine-linked glycosylation sites and 15 cysteine residues are present in the extracellular domain. This protein demonstrates two heptad arrays of leucine residues, with clustered cysteine residues, in a leucine zipper motif that may be involved in protein/protein interactions. There is a primary sequence similarity of the Kell glycoprotein with the neprilysin family of zinc-binding neutral endopeptidases such as bradykinin, neurotensin, enkephalin, oxytocin, and angiotensins I and II.

Biochemically, the monospecific anti-Kell antibodies precipitate immunologically a single 93 000 Da red cell membrane protein with two different Kell epitopes. This polypeptide exists as part of a large complex (from 115×10^3 Da to 200×10^3 Da in size) under non-reducing conditions. The Kell glycoprotein appears to exist as a homodimer in the membrane.

5.3.6

Lewis Blood Group

The Lewis antigens (ISBT No.007: Lewis/LE) expressed in red cells are unique, because these antigens themselves are not synthesized by erythroid precursors. These antigens are Lewis-active glycosphingolipid molecules basically present in plasma, and are adsorbed by the red cell membrane through an apparently passive process [20, 68].

Two forms of the Lewis antigens (Le^a and Le^b) are known as complexes with low- and high-density lipoproteins, and also as aqueous dispersions in the plasma (Table 5.1). There are approximately $4.5\text{--}7.3 \times 10^3$ Le^a molecules per red cell. The molecule associates with red cell membranes through the ceramide moiety.

The Lewis antigens are absent in red cells of newborns, and appear approximately 10 days after birth. The full activity of the Lewis antigens (Le^a) in red cells is established at approximately 24 months of age.

The Le^a and Le^b antigens are synthesized by two distinct fucosyltransferases under the control of the *Le* blood group gene and the *Se* blood group gene. The *Le* gene corresponds to an α (1, 3/1, 4) fucosyltransferase gene which is an *Fuc-TIII* or *FUT3* gene [69, 70, 80]. The enzyme encoded by this gene can use oligosaccharide precursors, including unsubstituted type 1 oligosaccharide precursors, to produce the Le^a antigen, and type 1 H antigens to generate Le^b antigens. *Le* gene-dependent expression of Le^a and Le^b molecules is identified at the epithelia lining the respiratory tract, urinary tract, digestive tract, salivary glands, and bile ducts [69, 70, 73, 80]. These tissues correspond to the tissue types capable of

expression of type 1 H molecules, the synthesis of which is determined by the *Se* locus.

α (1,3/1,4) Fucosyltransferase (FUT3) is a 363 amino acid-long type II transmembrane glycoprotein, which is composed of a 15 amino acid-long NH_2 -terminal cytosolic segment, a 19 residue transmembrane segment, and a 320 amino acid-long COOH-terminal catalytic segment, which is located in the Golgi apparatus. The enzyme produces several types of α (1,3) – and α (1,4) fucosylated oligosaccharides, which are Le^a , Le^b , Le^x , and Le^y molecules, and sialylated forms of the Le^a and Le^x antigens. The *FUT3* gene, as a member of an α (1,3) fucosyltransferase gene family, is located on chromosome 19p13.3.

The Lewis α (1,3/1,4) fucosyltransferase can use the oligosaccharide products formed by the *Se*-determined α (1,2) fucosyltransferase. In addition, the *Se* and *Le* fucosyltransferases are expressed in many of the same tissues. Therefore, the genotype at these two genes determines which of the Lewis-active oligosaccharide molecules is constructed. In secretor-positive subjects, type 1 oligosaccharide precursors are first converted into type 1 H molecules, which become substrates for the Lewis locus-encoded α (1,3/1,4) fucosyltransferase [70, 80]. This enzyme converts these into Le^b -active molecules: that is, an *Le* (a–b+) phenotype. On the other hand, in non-secretors, type 1 H antigens cannot be produced in secretory epithelia. Thus, these unsubstituted type 1 molecules are converted into Le^a -active oligosaccharides by the *Le*-encoded α (1,3/1,4) fucosyltransferase: that is an *Le* (a+b–) phenotype. In homozygotes for null alleles at the *Le* gene, the phenotype should be *Le* (a–b–). In this case, two possibilities exist. In Lewis-negative and secretor-positive subjects, type 1 H determinants produced remain unconverted into Le^b antigens. In Lewis-negative, secretor-negative subjects, the type 1 precursors remain unsubstituted by either blood group fucosyltransferase.

5.3.7

Duffy Blood Group

There are two major alleles in the Duffy blood group system (ISBT No.008: Duffy/FY), that is, Fy^a and Fy^b with virtually equivalent frequencies (Table 5.1). Other alleles are Fy^x with a weakly reactive form of Fy^b , *Fy* of a null allele, which produces no antigenic activities of Fy^a and Fy^b [20, 68, 97].

The Duffy antigen is a 338 amino acid-long polypeptide with seven membrane-spanning segments. The Fy^a and Fy^b antigens are localized on a glycoprotein with a molecular weight of from 38×10^3 to 90×10^3 Da. A significant amount of this molecule corresponds to asparagine-linked oligosaccharides, which demonstrate the heterogeneous migration properties in its native condition. The Fy^a and Fy^b alleles differ only by a single amino acid substitution at codon 44: a glycine for the Fy^a antigen, and aspartic acid for the Fy^b antigen.

Functionally, this protein corresponds to the red cell chemokine receptor, known as DARC (Duffy antigen receptor for chemokines). DARC removes excessive chemokines from the blood and tissues. In the homozygotes for the *Fy* allele, DARC expression in the bone marrow is defective, in spite of the normal expression of

DARC in the extra-marrow tissues. This polymorphism in the tissue-specific expression of DARC is accounted for by a single base pair difference between the $F\gamma$ allele and the $F\gamma^a$ or $F\gamma^b$ alleles [98, 99]. The sequence change is located in the promoter region of the *DARC* gene, and in the $F\gamma$ allele, leading to the disruption of a binding site for the erythroid lineage-specific transcription factor GATA-1.

Duffy antigens are important for the invasion of human red cells by *plasmodium vivax*. Successful invasion of the merozoite into the red cells requires the subsequent formation of a junction between the apex of the merozoite and the red cells. Formation of this junction and penetration of the merozoite into the red cells only occur on Duffy-positive red cells [100].

5.3.8

Kidd Blood Group

The Kidd antigen of the Kidd blood group system (ISBT No.009: Kidd/Jk) is a 46 000 to 60 000 Da protein [20, 68]. There are approximately 14 000 Kidd molecules in the red cells (Table 5.1). Two antigens (Jk^a and Jk^b) are expressed in their virtually equal gene frequencies, that is, 0.514 for the JK^a allele and 0.486 for the JK^b allele. A single amino acid substitution accounts for the polymorphism in the Kidd blood group system, that is, Asp at codon 280 of the *Kidd* gene for Jk^a , and Asn at the same codon for Jk^b , respectively. Approximately half of normal individuals express the Jk (a+b+) phenotype in the red cells, owing to the genotype $Jk^a Jk^b$. A quarter of individuals (26 % or 24 %) exhibit the Jk(a+b-) phenotype due to the genotype $Jk^a Jk^a$, or the Jk (a-b+) phenotype due to the genotype $Jk^b Jk^b$, respectively. The Jk (a-b-) phenotype is rarely observed. The gene mutation of aberrant splicing in the *Kidd* gene leads to the Kidd null phenotype.

It has been shown that the primary sequence of a human red cell urea transporter corresponds to the Kidd antigen [101].

5.3.9

LW Blood Group

There are two major allelic antigens with the LW blood group (ISBT No.016: Landsteiner-Weiner/LW), that is, LW^a and LW^b [20, 68, 102]. The LW^a antigen predominates and the LW^b antigen is detected in less than 1 % of the total population (Table 5.1). The two alleles yield the common phenotype LW (a+b-), the much less common phenotypes LW (a-b+) and LW (a+b+), and the rare phenotype LW (a-b-), which is homozygous for null alleles at the *LW* locus. Expression of the LW polypeptides is dependent on expression of the Rh polypeptides. There are approximately 4400 LW molecules per red cell.

The LW antigen corresponds to a 42 kDa red cell glycoprotein with a deglycosylated molecular mass of 25 000 Da. The COOH-terminal region of the LW glycoprotein is present at the surface of the red cells. Two structurally distinct LW proteins are present. The first one is a type I transmembrane protein with a short

cytoplasmic tail. Another one is a molecule without the membrane-spanning and cytoplasmic regions of the first longer form. The LW antigens are also one of the ICAM (the intracellular adhesion molecule) family, such as ICAM-4 [103]. This protein shares approximately 30 % of the same identity of the protein sequence with other members (ICAMs-1, 2, and 3).

The *LW* gene corresponds to three exons of 2.65 kb on human chromosome 19 [104]. The *LW^a* and *LW^b* alleles are different at a single base pair, in a codon 70 corresponding to one amino acid residue, that is, glutamine for *LW^a*, and arginine for *LW^b*. One *LW* null allele is known with a 10 base pair deletion, resulting in a truncated protein at a position proximal to its transmembrane and cytosolic regions.

5.3.10

Ii Blood Group

The commonly expressed antigen of the Ii blood group system is denoted I, and its absence is called i [20, 68, 105, 106] (Table 5.1). The Ii antigens are carbohydrate molecules. I activity corresponds to branched oligosaccharide structure formed by an N-acetylgalactosamine unit attached in β 1,6 linkage to a galactose residue within linear lactosamine polymers, whereas molecules with i reactivity correspond to oligosaccharide chains containing at least two repeating N-acetylgalactosamine units. It is known that oligosaccharide chains in neonatal red cells are unbranched, and that those in adult red cells are branched.

Red cell expression of the Ii blood group system is developmentally regulated. I determinants are deficient in embryonic, and cord red cells, which are highly reactive with anti-i antibodies. During the first 18 months after birth, this relationship is reversed to yield red cells with increased anti-I reactivity and diminished i reactivity, which is usually observed in adult red cells. This phenomenon corresponds to the fact that the increase in the I reactivity and the decrease in the i reactivity during early infancy are associated with the elaboration and display of increasing numbers of β 1,6-linked lactosamine units. I reactivity is determined by a locus encoding an N-acetylglucosaminyltransferase that is expressed in a developmentally regulated fashion.

The functions of the Ii blood group system are yet to be defined.

5.3.11

The Diego and Wright Blood Group Antigens on Band 3

Band 3 (anion exchanger 1: AE1) in red cells demonstrates several polymorphic peptide epitopes. The two major ones are the Diego blood group system (ISBT No.010: Diego/DI) and the Wright blood group system [20, 68] (Table 5.1).

The *Diego* (*Di^a*) allele occurs relatively frequently in the Japanese population, but is rare in Caucasians. There are two Diego antigens, i. e., *Di^a* and *Di^b*. The *Di^a* antigens correspond to a proline at position 854 of the 911 amino acid-long glycoprotein (band 3), whereas the *Di^b* antigens correspond to a leucine residue at the same position 854 of this molecule [107].

The *Wright* alleles correspond to the antigens Wr^a and Wr^b . The frequency of the Wr^a allele is rare, but the Wr^b allele is fairly prevalent. The Wr^a antigens correspond to a lysine residue at codon 658 of the AE1 gene, and the Wr^b antigens to a glutamine residue at the same codon 658, respectively [108]. Wr^b expression is suppressed in glycophorin A deficiency.

Seven minor antigens are present on band 3, these are: Waldner (Wa^a), Redelberger (Rb^a), Traversu (Tr^a), Wulfsberg (Wu), Moen (Mo^a), ELO, and Warrior (WARR). Amino acid substitutions at codon 552 (Thr → Ile), and at codon 565 (Gly → Ala) of the band 3 gene are known as WARR and Wu, respectively. Similarly, amino acid substitutions at codon 432 (Arg → Trp), at codon 548 (Pro → Leu), at codon 551 (Lys → Asn), and at codon 557 (Val → Met) correspond to ELO, Rb^a , Tr^a , and Wd^a antigens, respectively.

5.3.12

Other Minor Blood Group Antigens

The Chido (Ch) blood group system and the Rodgers (Rg) blood group system are known to be complementary-associated blood group antigens (Table 5.1).

The decay-accelerating factor (DAF) is one of the complementary regulatory proteins associated with red cell membranes as a glycosylphosphatidylinositol (GPI)-anchor protein. DAF-associated antigens are termed Cromer (Cr)-related antigens. The rare Inab phenotype corresponds to a total deficiency in the Cromer-related antigens (Table 5.1).

The Knops (Kn^a) antigens in red cell membranes are expressed by the complementary receptor 1 protein (CR 1) (Table 5.1).

The Cartwright (Yt^a and Yt^b) blood group system is associated with acetylcholinesterase in red cells, which is also a GPI-anchor protein. The type III red cells of paroxysmal nocturnal hemoglobinuria is deficient in acetylcholinesterase activity, demonstrating the Cartwright null phenotype (Table 5.1).

The Indian (In^a and In^b) antigens are expressed on CD44, which is the red cell isoform of the hyaluronan-binding protein (Table 5.1).

The Colton (Co^a and Co^b) blood group antigens are carried on an aquaporin-1, which is a red cell glycoprotein (Table 5.1).

The physiological relevance of these antigens remains to be clarified [20, 64, 68].

5.4

Glycosyl Phosphatidylinositol (GPI) Anchor Proteins

Glycosylphosphatidylinositol (GPI) anchor proteins are proteins with complex glycolipid structures, which are highly conserved in all eukaryotic cells [109–111]. The common core region consists of a molecule of phosphatidylinositol (PI) to which are attached four sugars, that is, one molecule of N-glucosamine and three molecules of mannose (Fig. 5.5). The last mannose is attached to the carboxyl end of the protein through phosphoethanolamine. A palmitoyl residue is added to the inositol

but may later be removed. The N-glucosamine is derived from the N-acetylglucosamine that is added and then deacetylated. The mannose residue is derived from dolichyl phosphoryl mannose.

GPI anchor biosynthesis starts on the cytoplasmic side of the rough endoplasmic reticulum [112]. N-Acetylglucosamine is transferred to a phosphatidylinositol acceptor. This product is deacetylated to form glucosamidyl phosphatidylinositol. The first mannose is derived from dolichol phosphoryl mannose, as described above. The phosphoethanolamine is added. Addition of the GPI anchor precursor to the carboxy-terminus of the protein occurs on the luminal side of the endoplasmic reticulum, after amino acid residues 17 to 31 have been cleaved from the protein. When the synthesis of the anchor is completed, it proceeds through the Golgi apparatus and on the membrane surface.

There are many membrane proteins which are related to GPI-anchor proteins, such as: (1) enzymes; acetylcholinesterase and leukocyte alkaline phosphatase, (2) complementary defense proteins; decay accelerating factor (DAF and CD55), a membrane inhibitor of reactive lysis (MIRL, CD59, protectin), and C8-binding protein (homologous restriction factor), (3) immunologic proteins; Fc γ receptor IIIa, lymphocyte function-associated antigen-3 (LFA-3, CD58), endotoxin-binding protein receptor (CD14), and CD_w52 (Campath-1), (4) receptors; urokinase (plasminogen activator) receptor, and folate receptor, and (5) granulocyte proteins of unknown function; CD14, CD48, CD66, and Dombrock-Holley/Gregory-bearing

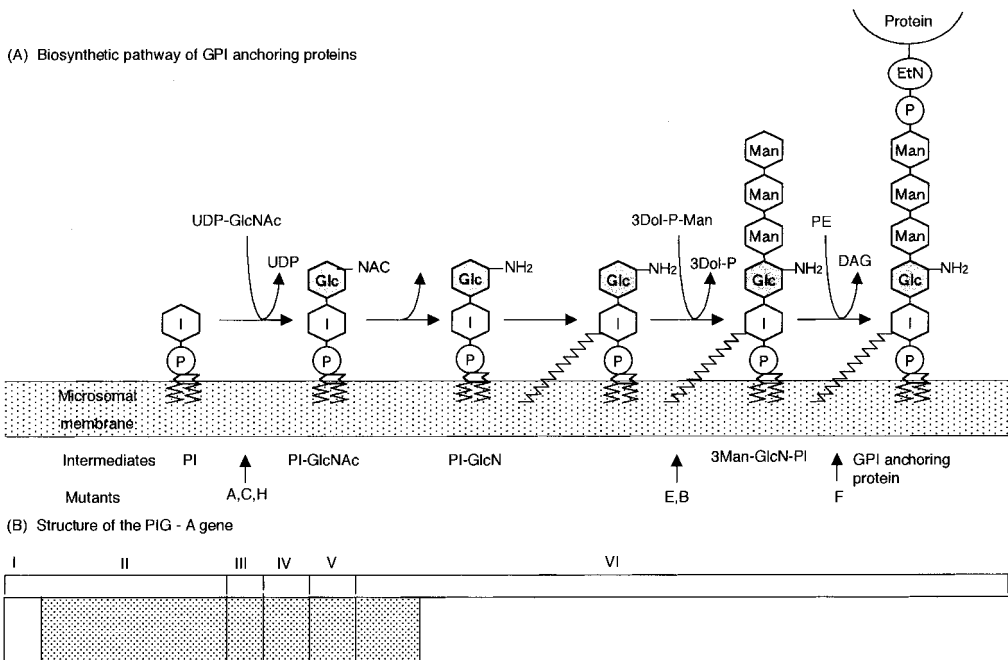


Figure 5.5 Biosynthetic pathway of glycosyl phosphatidylinositol (GPI)-anchoring proteins (A), and the molecular structure of the *PIG-A* gene (B).

500bp

protein. It is known that these proteins are missing from the blood cells in paroxysmal nocturnal hemoglobinuria (PNH), in which PNH blood cells are unable to synthesize the mature GPI anchor precursors. In lymphoblastoid cells of PNH, the block always occurs at the step when N-acetylglucosamine is transferred from UDP-N-acetylglucosamine to phosphatidylinositol. This is the first step of the pathway and is catalyzed by the α 1,6-N-acetylglucosaminyltransferase, which is an enzyme complex formed by four gene products, i.e., *PIGA*, *PIGC*, *PIGH*, and *hGP1* [113]. Of these, the *PIGA* gene has been identified as pathognomonic for PNH [114]. The *PIGA* (phosphatidylinositol glycan complementation group A) gene consists of six exons and encodes a putative protein of 484 amino acids (approximately 60 kDa). This gene is located on chromosome Xp22.1. Many mutations of this gene have been reported in PNH patients. Recent advances on PNH and related disorders have recently been reviewed in detail [115].

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6

Anchoring Proteins

6.1

Ankyrin

6.1.1

Introduction

Ankyrin is one of the major proteins of human red cells, making up approximately 5 % of the total membrane proteins [1–3]. Ankyrin is a large, 206 kDa sulfhydryl-rich protein with a molecular size of 8.3×10 nm. It is present at a level of 120×10^3 copies per cell (Tables 1.1 and 1.2).

Functionally, ankyrin is connected with β -spectrin through a high affinity linkage (K_d : $\sim 10^{-7}$ M), and with the cytoplasmic domain of band 3, which also has a high affinity linkage (K_d : $\sim 10^{-7}$ to 10^{-8} M) [4, 5]. Ankyrin is a polar protein, and is involved in the local segregation of integral membrane proteins. The polarization of membrane proteins appears to be produced by the relative affinities of the various isoforms of ankyrins for target membrane proteins. These ankyrin isoforms appear to be expressed through tissue-specific, developmentally-regulated control [6–10], as discussed below.

6.1.2

Structure of Red Cell Ankyrin

Ankyrin is composed of three domains [6, 7, 11–13]: (1) a membrane (band 3)-binding domain (89 kDa) at its NH_2 -terminal (amino acids 2 to 827), (2) a spectrin binding domain (62 kDa) at the central part of the molecule (amino acids 828 to 1382), and (3) a regulatory domain (55 kDa) at its COOH -terminal (amino acids 1383 to 1881) (Fig. 6.1). The NH_2 -terminal band 3-binding domain is basic, the central spectrin-binding domain is neutral, but heavily phosphorylated, and the COOH -terminal regulatory domain is highly acidic.

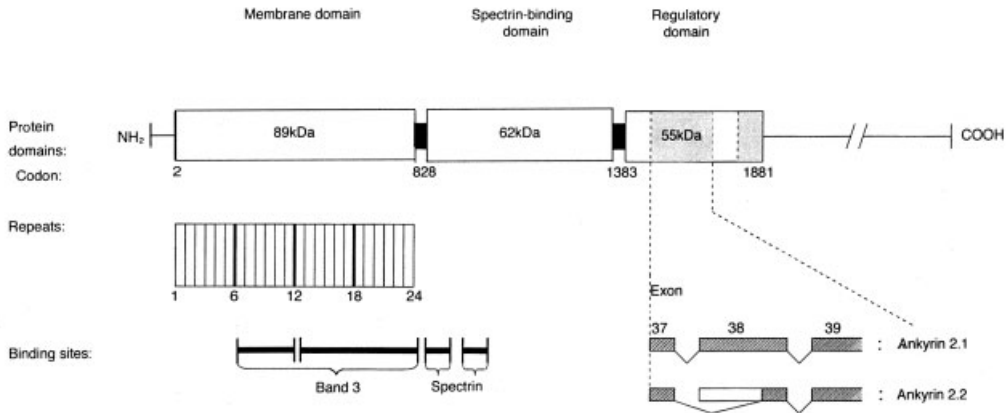


Figure 6.1 Structure of red cell ankyrin. Protein domain structure of erythroid ankyrin and its binding sites are shown schematically. Genetic basis of ankyrin 2.1 and 2.2 is also shown at the lower-right.

6.1.2.1 Membrane (Band 3)-Binding Domain of Ankyrin

This 89 kDa domain, which is at the NH_2 -terminal portion of the ankyrin molecule, is almost entirely composed of 24 consecutive 33 amino acid tandem repeats (so-called “cdc 10/ankyrin repeats”, or “ankyrin repeats”), which are subdivided into six repeat folding units [12, 13]. These repeats are fairly similar to each other.

Binding sites for band 3 and at least six other types of integral membrane proteins are present in this domain. Repeats 7 through 12 (folding unit 2) and especially repeat 13 through 24 (folding units 3 and 4) form two distinct but cooperating binding sites for band 3. These two binding sites of ankyrin for band 3 are able to interact with four band 3 molecules, because normally band 3 is present in a dimeric form on the membrane. This binding of ankyrin to band 3 appears to be critical for maintaining the normal integrity of membrane functions, especially membrane stability, because selective disruption of the ankyrin/band 3 interaction in intact red cells, which are placed at a slightly alkaline pH, decreases membrane stability markedly.

The 15 out of 33 amino acids in the ankyrin repeats are highly conserved. Their conserved sequence is “-G-TPLH-AA-GH—V(or A)—LL-GA-N(or D)—”. L-shaped structures of the ankyrin repeats are composed of a pair of α -helices that form an antiparallel coiled-configuration, which is followed by an extended loop perpendicular to the helices and a β -hairpin [8, 14–19]. Very similar repeats have commonly been found in various proteins, in all phyla. Therefore, the ankyrin repeats appear to have been propagated as one of the versatile modules for specific ligands during evolution.

6.1.2.2 Spectrin-Binding Domain of Ankyrin

This spectrin-binding domain is also known as the central domain, and was previously known as the 62 kDa domain. The spectrin-binding sites are located in

the beginning and middle regions of this domain. The site in the middle portion is highly conserved and appears to be the principal area of binding [20]. These regions are found to be those near the end of the β -spectrin molecule (repeats 15 and 16) which are involved in dimer–tetramer self-association [21]. Although two binding sites for spectrin are available in the ankyrin molecule, each spectrin tetramer appears to bind only one ankyrin molecule, probably because ankyrin is able to bind to the spectrin tetramer approximately ten-fold more strongly than to the spectrin dimer.

6.1.2.3 Regulatory Domain of Ankyrin

This domain (55 kDa) is at the COOH-terminal region of the ankyrin molecule, and is known to contain regulatory sequences that enhance or reduce the extent of interaction of ankyrin with spectrin and band 3. Ankyrin binds to the COOH-terminal region of the β -spectrin with its 55 kDa domain and to the cytoplasmic tail of band 3 via repeated sequences in the NH₂-terminal 89 kDa domain. The regulatory domain consists of multiple isoforms of different sizes and functions, which are produced by alternative splicing [8, 9]. One of these distinct isoforms is ankyrin 2.2, which lacks the acidic 162 amino acid sequence from exon 38, which is found in full-sized ankyrin (protein 2.1) [6]. This protein 2.2 (the smaller isoform: ankyrin 2.2) is an activated ankyrin, and enhances ankyrin binding to band 3 and spectrin. Use of alternative promoters has recently been shown to produce a muscle-specific, truncated isoform of ANK 1 [9].

Many alternatively spliced isoforms of ankyrin at the three COOH-terminal exons are known, these are isoforms lacking: (1) exons 38 and 39, (2) exons 36 through 39, and (3) exons 36 through 41. The COOH-terminal exons are highly conserved [8].

6.1.3

Functions of Ankyrin

The major function of ankyrin lies in its binding to β -spectrin and band 3 [22]. These bindings create a tight association between spectrin and band 3, but the strength of binding can be modified by the extent of phosphorylation [23]. The presence of seven phosphorylation sites have been proven *in vitro* for casein kinase I and cyclic AMP-independent protein kinase. Unphosphorylated ankyrin binds preferentially to spectrin tetramers and oligomers rather than to spectrin dimers, but phosphorylation removes this preference. Phosphorylation also reduces the capacity of ankyrin to bind band 3. In addition, ankyrin is phosphorylated by protein kinase A.

Stoichiometrically, one ankyrin molecule links each spectrin tetramer to the membrane. Since these binding interactions are cooperative, attachment of ankyrin to band 3 enhances greatly the ability of the molecule to organize the spectrin, to which it is attached, into tetramers. It is also true that spectrin binding enhances the affinity for band 3. Therefore, ankyrin plays a crucial role in the organization of the network.

6.1.4

Erythroid and Nonerythroid Ankyrins

Ankyrins are widely expressed, and several ankyrin gene families are known [2]. Red cell ankyrin (ankyrin_R, or ANK 1) is expressed not only in red cells, but also in myocytes, endothelial cells, and brain (especially in Purkinje cells of the cerebellum) [6, 7]. The gene is located on 8p11. 2. Ankyrin_B (ANK 2) is a neural form, and is present in neuronal cell bodies and dendrites [24, 25]. The gene is located on 4q25–q27. Ankyrin_C (ANK 3) is the most widely distributed, mostly in epithelia and axons, but also in megakaryocytes, macrophages, myocytes, melanocytes, hepatocytes, kidney cells, and testicular Leydig's cells [26, 27]. Nonerythroid ankyrins interact with a variety of integral membrane proteins other than band 3 (AE1), such as Na⁺, K⁺-ATPase [28–30], the voltage-dependent axonal Na⁺ channel [31], the amiloride-sensitive epithelial channel [32], the cardiac Na⁺/Ca²⁺ exchanger [33], H⁺, K⁺-ATPase [34], the IP₃ receptor [35], CD44 [36], and a group of neurofascin-related brain adhesion molecules [37, 38]. Mice with targeted disruption of ANK 1 or ANK 3 exhibit neurological abnormalities, including Purkinje cell degeneration and ataxia, and those with targeted disruption of ANK 2 also exhibit brain defects, but with more clear-cut abnormalities in brain development [39].

6.2

Protein 4.2

Red cell protein 4.2 (P4.2) is one of the major components of the red cell membrane skeletal network, which binds to the cytoplasmic domain of anion exchanger band 3 and interacts with ankyrin in red cells [40–43]. Patients with P4.2 deficiency in their red cell membranes suffer from congenital hemolytic anemia with microspherocytosis or other such disorders. This fact suggests that P4.2 plays an important role in maintaining the stability and flexibility of red cells. P4.2 has been reviewed previously by two authors in 1993 [40], 1994 [41, 42] and recently in 2000 [43].

6.2.1

Protein Chemistry of Protein 4.2

Protein 4.2 is a membrane protein (Tables 1.1 and 1.2, Fig. 6.2) accounting for approximately 5 % of the total membrane protein content and for 250×10^3 copies per red cell. It has a molecular weight of 72 kDa on SDS–PAGE [44, 45].

Extraction of protein 4.2 from red cell membranes is more difficult than for any of the other peripheral proteins, even under high and low ionic strength conditions. Therefore, strong basic conditions (pH 11 or above) have been used in combination with gel filtration with 1 M KI-Sephacryl S-200 as the standard method for the extraction of protein 4.2. This procedure yields 1–2 mg of “type I” protein 4.2 from 500 mL of whole blood with a purity of approximately 85 %. This protein is

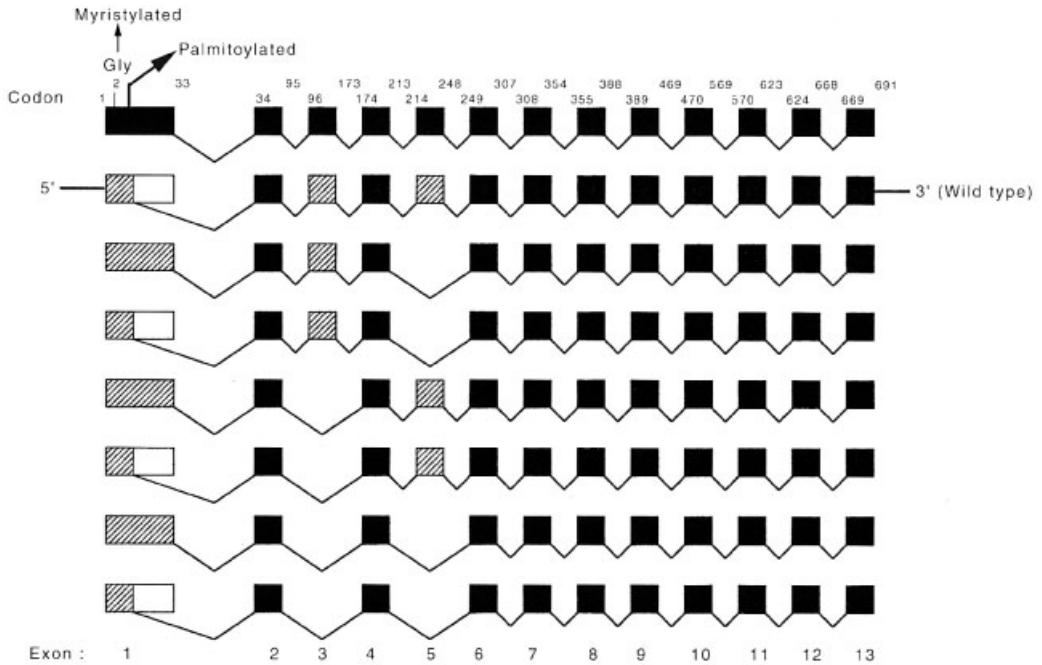


Figure 6.2 Molecular structure of protein 4.2 and the genetic isoforms. A wild type of protein 4.2 is shown at the second line from the top. Black solid boxes indicate constitutive coding exons, and shaded boxes show alternative coding exons.

water-soluble, and is difficult to separate from residual ankyrin and protein 4.1. The use of other extraction methods with 10 to 20 mM lithium diiodosalicylate, 6 mM 2,3-dimethylmaleic anhydride, 5 mM *p*-chloromercuribenzoic acid (pCMB), or 1 mM *p*-chloromercuribenzoic sulfate (pCMBS) have been reported. The non-ionic detergent Triton X-100 can also be used to extract protein 4.2 from red cell membranes. Under these conditions, band 3 is co-extracted along with the portion of protein 4.2, suggesting an association of protein 4.2 with band 3 in red cell membranes *in situ* [44, 45].

An alternative method using 2 M Tris-HCl (pH 7.6) has also been used to extract protein 4.2 with a purity of greater than 97%. However, this “type II” variety of protein 4.2 is less water-soluble and behaves like an integral protein. It has been speculated that its characteristic hydrophobicity is due to myristylation [46].

Protein 4.2 purified by the standard pH 11 method with 1 M KI-Sephacryl S-200 appears to be heterogeneous in size and probably primarily consists of a mixture of dimers and trimers. Electron microscopically, purified protein 4.2 appears as globular particles with diameters in the range 80–150 Å, and has been suggested as being tetrameric *in situ* in the membrane.

Protein 4.2 in human red cell membranes is known to be myristylated [47] at a site near the N-terminus, as assayed by the release of myristoyl glycine from partially hydrolyzed protein 4.2. Glycine at the second position appears to be respon-

sible for this myristylation (*N*-myristoyl glycine). Further studies of its biological functions should be considered.

It has also been reported that protein 4.2 is palmitoylated under physiological conditions [48]. After labeling of intact human red cells with [^3H] palmitic acid, radioactivity was found to be associated with protein 4.2 by immunoprecipitation of peripheral membrane proteins extracted at pH 11 from ghosts with anti-protein 4.2 antibody. The fatty acid linked to protein 4.2 was identified as palmitic acid. Protein 4.2 could be depalmitoylated with hydroxylamine, suggesting a thioester linkage. Depalmitoylated protein 4.2 showed significantly decreased binding to protein 4.2-depleted membranes, as compared with native protein 4.2. Several red cell membrane proteins including ankyrin, band 3, p55, protein 4.1 and spectrin were palmitoylated. Fatty acid acylation of proteins confers an extra hydrophobic moiety on proteins, which promotes hydrophobic protein–membrane and protein–protein interactions. Whereas control protein 4.2 showed a binding capacity of 280 mg per g of vesicle protein (band 3), depalmitoylated protein 4.2 showed a capacity of 108 mg per g of vesicle protein. Therefore, palmitoylation of protein 4.2 appears to favor its interaction with band 3 in the membrane.

To date, protein 4.2 has not been crystallized. Crystallography is thus expected to be performed in the future.

6.2.2

Functions of Protein 4.2

The major functions of protein 4.2 are, in conjunction with other membrane proteins, topographically adjacent to it *in situ* in red cell membranes, especially band 3, ankyrin, spectrin, and protein 4.1.

6.2.2.1 Binding Properties of Protein 4.2

Interactions of protein 4.2 with band 3 When Triton X-100 extracts of red cell membranes were fractionated by ion exchange chromatography and non-denaturing gel electrophoresis, protein 4.2 was found with band 3 [44]. In addition, direct binding assays have indicated that an excess of the cytoplasmic domain of band 3 eliminated the normal ($2\text{--}8 \times 10^{-7}$ M) binding of purified protein 4.2 to red cell inside-out vesicles (IOV). The binding of protein 4.2 to the purified cytoplasmic domain of band 3 usually takes from 6 to 20 h for complete saturation. Therefore, it has been hypothesized that re-binding of protein 4.2 to the membrane probably requires the formation of other types of associations apart from protein–protein contacts at the membrane–medium interface, perhaps the formation of protein 4.2 or band 3 oligomers. Although the major binding site of protein 4.2 has been considered to be the cytoplasmic domain of band 3, no direct evidence has been found, because the state of self-association of purified protein 4.2 is heterogeneous and the exact oligomeric state of band 3 *in situ* in membranes under the conditions of the binding assays is unknown. It has been tentatively estimated that the

stoichiometry of the protein 4.2 to band 3 interaction is approximately 1:3.9 on a monomer basis.

One synthetic peptide of protein 4.2 (P8: L⁶¹ FVRRGQPFTIILYF) was found to bind strongly to the cytoplasmic domain of band 3 [49]. Four other peptides (P22: L²⁷¹ LNKRRGSVPILRQW, P27: G³⁴⁶ EGQRGRIWIFQTST, P41: L⁵⁵⁶ WRKKLHLTLSANLE, and P48: I⁶⁶¹ HRERSYRFRSVWPE) bind less strongly. These peptides have in common a cluster of two or three basic amino acid residues (arginine or lysine) in a region with virtually no acidic residues. The cytoplasmic domain of band 3 bound in a saturated manner to P8 with a K_d of 0.16 μ M and a capacity of 0.56 mol of the cytoplasmic domain of band 3 monomer per mol of P8. Replacement of R⁶⁴R with R⁶⁴G, G⁶⁴R or G⁶⁴G almost completely abolishes the cytoplasmic domain of band 3 binding, suggesting that R⁶⁴R is essential for its binding. P8 competitively inhibits binding of purified human red cell P4.2 to the cytoplasmic domain of band 3.

Protein 4.2 can be found with band 3 and ankyrin in an immunoprecipitated complex, probably due to association of protein 4.2 with ankyrin and band 3 *in situ* [40]. A partial deficiency of band 3 has also been found to be accompanied by partial deficiency of protein 4.2 [43]. Furthermore, a cow with total band 3 deficiency and mice with the targeted band 3-knock-out gene clearly demonstrated a loss of protein 4.2 in their red cell membranes [43]. The rotational and lateral mobility of band 3 in red cell membranes from patients with protein 4.2 deficiency is substantially increased [50–52]. In our study with fluorescence recovery after use of the photobleaching method (FRAP), the immobile fraction of band 3, which constitutes about 60 % of the total in normal red cells, was totally absent in complete protein 4.2 deficiency. It is also interesting that, with complete deficiency of protein 4.2, the number of intramembrane particles (IMP) has been found to be reduced with a shift to larger sizes, indicating the possibility of increased oligomerization of band 3 molecules in these red cells [53]. Heat treatment considerably enhanced this effect. The structural and functional characteristics of band 3 in these protein 4.2-deficient red cells appeared normal in terms of the cleavage pattern of band 3 fragments and the binding properties of band 3 to protein 4.2 or ankyrin.

Interactions of protein 4.2 with ankyrin Considering the association of protein 4.2 with ankyrin, it has been reported that protein 4.2 can bind to 0.65 mol of ankyrin per mol of protein 4.2 with a K_d of from 1 to 3.5×10^{-7} M on the Scatchard plot *in vitro* [45]. However, this binding requires several hours to approach saturation in solution, and no conclusive evidence has yet been shown for an association of protein 4.2 with ankyrin in the membrane *in situ*. It has also been shown that ankyrin can bind to the cytoplasmic domain of band 3 in IOV without protein 4.2, and that reassociation of ankyrin with IOV is unaffected even when protein 4.2 is removed from the IOV.

In some hemolytic anemias, however, a partial deficiency of protein 4.2 has also been reported in ankyrin deficiencies [54]. Decreased protein 4.2 content has been noted in a mouse strain (nb/nb) with an ankyrin deficiency. In a protein 4.2 deficiency with the 142 Ala $\rightarrow$ Thr point mutation, it was reported that ankyrin was par-

tially released from the patient's red cell membranes upon preparation of IOV, suggesting that protein 4.2 might contribute to the stability of the membrane protein association.

It has been shown that the red cell membranes of nb/nb mice, which were almost completely deficient in full-length 210 kDa ankyrin due to a defect in the Ank-1 gene on mouse chromosome 8, were severely (up to 73 %) deficient in protein 4.2 content [55]. This deficiency of protein 4.2 in nb/nb homozygous mice was not the result of defective protein 4.2 synthesis. Reconstitution of nb/nb to inside-out vesicles with human red cell ankyrin restored ankyrin levels to up to 80 % of the normal levels and increased binding of exogenously added human red cell protein 4.2 by approximately 60 %. These results suggest that ankyrin is required for normal associations of protein 4.2 with the red cell membrane.

Interactions of protein 4.2 with spectrin Normal protein 4.2 has been shown to bind to spectrin in solution and to promote the binding of spectrin to ankyrin-stripped inside-out vesicles [51]. Two independent classes of binding sites of protein 4.2 to spectrin have been identified: 1) a high-affinity (a binding coefficient: $K_d = 7.4 \pm 0.2 \times 10^{-9} \text{ M}^{-1}$), low-capacity ($0.6 \pm 0.8 \times 10^{-9} \text{ M L}^{-1}$) class of sites; and 2) a low-affinity ($K_d = 2.8 \pm 2.0 \times 10^{-7} \text{ M}^{-1}$), high capacity ($5.8 \pm 1.0 \times 10^{-9} \text{ M L}^{-1}$) class of sites. It has been calculated that, at saturation, there is approximately one spectrin binding site per seven protein 4.2 molecules. Therefore, protein 4.2 provides low-affinity binding sites for both band 3 oligomers and spectrin dimers on the human red cell membrane. These observations suggest that protein 4.2 may stabilize skeleton–membrane interactions by providing a direct link between band 3 and spectrin. A spectrin-binding domain of human erythrocyte membrane protein 4.2 has recently been identified [56].

In a disease state, i. e., in red cells with total protein 4.2 deficiency and also in red cells of 4.2^{-/-} mice, the cytoskeletal proteins (spectrins, ankyrin, and protein 4.1) are not deficient [41, 42, 57]. However, the cytoskeletal network in these protein 4.2-deficient red cells appears to be less extended when studied by electron microscopy using the surface replica method and the quick-freeze deep-etching method [53]. Interestingly, the cytoskeletal network in these protein 4.2-deficient red cells becomes markedly disorganized, with the appearance of larger aggregates when heat-treated up to 48 °C [50]. Under these conditions, a marked decrease in red cell membrane deformability has been observed by ektacytometry. It should be noted that the spectrin and ankyrin contents were maintained as normal in these cells. Therefore, these abnormalities appear to be independent of spectrin and ankyrin *per se*, and due to the lack of protein 4.2. These results raise the possibility that protein 4.2 may play a role in connecting the cytoskeletal network to integral proteins (especially band 3) as a type of anchoring protein.

Interaction of protein 4.2 with protein 4.1 As to a possible association of protein 4.2 with protein 4.1, it has been reported that protein 4.1 may interact with protein 4.2 in solution, suggesting the masking of the binding domains of these proteins when they are present together in solution [45]. Protein 4.2, protein 4.1 and

ankyrin binding are partially inhibited (about 50%) by the presence of these proteins.

The protein 4.1 and protein 4.2 binding sites are localized at the nearby sites on the cytoplasmic domain of band 3. It is possible that, in the absence of protein 4.2, additional binding sites for protein 4.1 on band 3 may be exposed. The content of protein 4.1, however, remains nearly normal both in human red cells when there is a total deficiency of protein 4.2 and in 4.2^{-/-} mouse red cells [41, 42, 57].

It is also worth noting that the content of protein 4.2 appears to be normal in mice with a complete deficiency of all protein 4.1 R isoforms, which had been generated by gene knock-out technology [58].

6.2.2.2 Transglutaminase Activity of Protein 4.2

Protein 4.2 in human red cells has no transglutaminase activity, although the homology in the gene structure between protein 4.2 and transglutaminase is high (i.e., an overall identity of 32% in a 446 amino-acid overlap with guinea-pig liver transglutaminase, and of 27% in a 639 amino-acid overlap with human coagulation factor XIII subunit a) [59]. It has been speculated that the lack of transglutaminase activity may be due to the presence of an alanine substituted for the cysteine at the active site of the molecule.

6.2.2.3 Phosphorylation of Protein 4.2

Although phosphorylation has not been observed on protein 4.2 extracted from mature human red cells, protein 4.2 can be phosphorylated, if it is purified by methods that result in exposing phosphorylation sites through alteration of protein 4.2 sulfhydryl groups [40, 46]. Seventeen potential phosphorylation sites have been activated in red cell ghosts, i.e., eight possible protein kinase C sites, seven casein kinase II sites, one tyrosine kinase site, and one cAMP- (or cGMP-) dependent kinase site [47]. It has been suggested that membrane-associated protein 4.2 in human mature red cells is already fully phosphorylated with little or no turnover under normal conditions. The physiological functions of such potential protein 4.2 phosphorylation is unknown.

The activities of the major red cell kinases have recently been determined to assess the phosphorylation status in red cells of 4.2^{-/-} mice [57]. Cytosolic protein kinase C (PKC) was significantly decreased with decreased PKC- α and PKC- β I isoforms and normal PKC- β III in 4.2^{-/-} red cells. Cytosolic protein kinase A (PKA) activity was increased in these red cells. Basal phosphorylation was increased and PMA-stimulated phosphorylation was reduced in 4.2^{-/-} red cells. Cytosolic casein kinase I (CK I) activity was normal, but cytosolic CK II activity was decreased in these red cells. The functional significance of these activities remains to be clarified at some point in the future.

6.2.3

Protein 4.2 in Red Cell Membrane Ultrastructure

Although the localization of P4.2 in the red cell membrane structure has still not been elucidated in detail, there are several positive pieces of evidence. With a total deficiency of P4.2, it is now recognized that the intramembrane particles (IMPs) are clustered in the inside-out vesicles (IOVs) of the patient's red cells [41, 50, 53]. In addition, electron microscopic studies with the freeze fracture method have shown IMPs on the red cell ghosts to be enlarged, suggesting increased oligomerization of band 3 molecules. This phenomenon has been verified by biophysical analyses [41–43, 53]. Furthermore, analyses with the quick-freeze deep-etching method or the surface replica method have shown the cytoskeletal network to be disrupted in this disorder [41–43, 53]. These results appear to indicate that P4.2 molecules are located near band 3 molecules and membrane proteins making up the cytoskeletal network. Biophysical analyses, especially with ektacytometry, have revealed the increased instability of the cytoskeletal network of the patient's red cells. It has recently been proven that P4.2 can bind directly to spectrins [51], implying that P4.2 may play an important role as one of the anchoring proteins connecting the cytoskeletal network to the integral proteins (particularly band 3 molecules).

The exact location of various membrane proteins *in situ* in the normal human red cell membrane ultrastructure has been studied by immuno-electron microscopy with the surface replica method by utilizing antibodies against various membrane proteins: i.e., spectrins, ankyrin, band 3 (the cytoplasmic domain), protein 4.1 and protein 4.2. At first, spectrins were readily detected as major constituents of the cytoskeletal network, and ankyrin and protein 4.1 were also identified on it. Band 3 molecules were found attached to the cytoskeletal network as an immobile form of band 3. Other band 3 molecules were located inside the basic units, as the mobile form of band 3. P4.2, however, could not be detected by this procedure, when the antibody-conjugated immunogold particles were applied to the open red cell ghosts, implying that the epitopes of P4.2 were not exposed. Therefore, normal red cell ghosts were subjected to gentle treatment with Triton X-100 to remove part of the cytoskeletal network. P4.2 was then found to be attached predominantly to the cytoskeletal network. These results suggest that P4.2 is very likely to be present at the outer face of the cytoskeletal network and even under the lipid bilayer, and is attached to spectrins and band 3, as shown by biophysical studies [51].

6.2.4

Protein 4.2 Gene**6.2.4.1 Characteristics of Genomic DNA**

The human red cell protein 4.2 gene is ~20 kb in length and contains 13 exons and 12 introns [59–63]. The coding sequence from the genomic DNA is identical to the cDNA sequence. Nucleotide polymorphism has been observed in the normal

human protein 4.2 gene. The exons range in size from 104 to 314 base pairs with an average size of 170 base pairs, while the introns vary from 6.4 to 0.3 kb. All the exon–intron boundaries follow the consensus 5' donor–3' acceptor splice junction sequence for eukaryotic genes of gt-ag. The gene is localized at human chromosome 15q15–q21 [62, 63].

The upstream region of the protein 4.2 gene contains several elements that are similar in sequence to the upstream elements of the genes for β -globin and porphobilinogen deaminase, which are also red cell protein genes [59, 62]. The elements are spaced a similar distance from the transcription start site and have similar relative spacings and orders. These similarities have made it easier to identify five possible regulatory cis-elements in the protein 4.2 gene, starting –20 nucleotides upstream from the transcription start site, i. e.: (1) a possible TATA element, (2) a short G + C-rich domain, which could be an Spl binding site, (3) a possible CAAT box, (4) a CAAC box, and (5) two GF-1 binding domains, one at –23 to –28, and another one at –173 to –178 [59]. These findings suggest the use of common cis-elements in these three erythroid genes, although the identification of these elements as having a regulatory function in protein 4.2 gene expression is highly speculative. It has also been reported [62] that the nucleotides upstream from the cDNA start site (nt 1) are: (1) CAGT (nt –4 to –1), agreeing well with the CA cap signal; (2) nucleotides –26 to –21 upstream from the cDNA start site having a sequence of ATAAAA, which agrees well in sequence and position with the promoter TATA box for eukaryotic genes; (3) a CCAT sequence was noted at nt –89 to –86 within the reported –385 nt upstream sequence, where upstream promoter elements are located; (4) a GC-rich region from nt –85 to –34 (G/C to A/T ratio = 3); (5) within this region, a sequence of CCCACCCC CTCCCCC containing a CACC element (nt –83 to –80) that is a potential binding site for the Spl nuclear factor; and (6) two AGATAA sequences for potential binding of erythroid-specific transcription factor GATA-1 (also known as GF-1, NF-E1, Eryf-1) located at nt –175 to –170 and at nt –28 to –23, respectively. The number of the 5'-CpG-3' dinucleotide sites appears to be small, unlike the β -spectrin gene that has numerous 5'-CpG-3' sites known as the so-called "CpG islands" [64]. The 5'-CpG-3' sites of the protein 4.2 gene were highly methylated, when genomic DNA was prepared from mononuclear cells in normal human peripheral blood.

Alignment of the protein 4.2 amino acid sequence with that of a subunit of human coagulation factor XIII and division of the sequence into exons have revealed a remarkable correspondence, although the gene for the a subunit of human factor XIII, which is on chromosome 6p24–p25, is 160 kb and has 15 exons and 14 introns, while the gene for protein 4.2 is only 20 kb and contains 13 exons and 12 introns [59]. With only one exception, the exons of protein 4.2 are very similar and in many cases identical in size to the exons of the a subunit of factor XIII with which they are paired. In addition, in every case, the corresponding intervening introns are of the same splice junction class. These and other similarities suggest that the gene for protein 4.2 is closely related to and possibly derived from that for the a subunit of factor XIII and that the proteins may share common structural and functional properties. However, it should be noted that, despite

this close similarity, purified protein 4.2 has no transglutaminase activity *in vitro*, and that normal red cell membranes do not contain transglutaminase activity. The lack of protein 4.2 transglutaminase activity is induced by the substitution of the cysteine (GQCWVF) at the highly conserved consensus sequence in the transglutaminase, which is substituted for an alanine (GQAWVL) in protein 4.2. The cysteine appears to be required for transglutaminase activity. It is also possible that the substitution of a leucine for a phenylalanine may also be responsible for a loss of this activity.

It has been shown that reticulocytes contain two forms of protein 4.2 mRNA, a small form (P4.2S) encoding a protein of 691 amino acids, and a larger form (P4.2L), which contains an additional 90 nucleotides following nucleotide +9, encoding a protein of 721 amino acids [59, 62]. Protein 4.2 exon I contains a 5' non-coding sequence, the translation start site, and 99 nucleotides encoding 33 amino acids. These 33 amino acids are identical to the first 33 amino acids of the larger protein 4.2 transcript. The last 90 nucleotides of exon I, coding for 30 amino acids, are removed by splicing in order to generate the smaller transcript, coding for the 691 amino acid protein (a wild type of protein 4.2: 72 kDa on the SDS-PAGE).

The genomic organization of the protein 4.2 gene of human red cells contains 13 exons, i.e.: exon I, ut ~33 residues; II, 34–95; III, 96–173; IV, 174–213; V, 214–248; VI, 249–307; VII, 308–354; VIII, 355–388; IX, 389–469; X, 470–569; XI, 570–623; XII, 624–668; and XIII, 669–ut (ut, untranslated sequence) [59, 60] (Fig. 6.2). The sizes of the introns were 6490 for intron 1, 900 for 2, 580 for 3, 340 for 4, 940 for 5, 320 for 6, 740 for 7, 500 for 8, 390 for 9, 2560 for 10, 2200 for 11, and 3080 for 12.

6.2.4.2 cDNA of the Protein 4.2 Gene

Protein 4.2 complementary DNA (cDNA) obtained from a human reticulocyte cDNA library has been cloned and sequenced [60–62]. The full-length cDNA was 2.35 kb and contained an open reading frame with a 227–nt untranslated region upstream from the putative ATG start codon. The calculated molecular weight was 76.9 kDa encoding 691 amino acids. The nucleotide sequence CAACCATGC around this initiation site was similar to the consensus sequence for initiation found in higher eukaryotes, except that the second nt in the P4.2 cDNAs was A rather than C [61]. The presence or absence of the 90 nt insert gave rise to two P4.2 cDNA sequences; that is, a P4.2S from 2073 bp and a P4.2L from 2163 bp [59, 62].

The amino acid sequence derived from the 2.5 kb cDNA contained ~43 % non-polar, ~35 % polar, ~10 % acidic, and ~12 % basic amino acid residues [61]. The most abundant amino acids were leucine (82 residues) and alanine (60 residues). There were 49 serine and 43 threonine residues, which are potential sites for O-glycosylation and represent 13 % of the total residues. There were 16 cysteine residues, six potential N-glycosylation sites (Asn-Xaa-Ser/Thr) at Asn-103, -420, -447, -529, -604, and -705, one potential cAMP-dependent phosphorylation site (basic-basic-Xaa-Ser) at Ser-278, and nine potential protein kinase C phosphoryla-

tion sites (Ser/Thr-Xaa-Arg/Lys) at Ser-7, -57, -58, -154, -224, -449, -455, and -666, and Thr-287 [61]. There was one Arg-Gly-Asp sequence at 518–520.

Secondary structure analysis predicted that P4.2 should contain ~3 % β -sheet, ~24 % α -helix, and ~45 % reverse turns. Hydropathy analysis of the deduced amino acid sequence revealed a major hydrophobic domain (residues 298–322), which was predicted to be mainly a β -sheet structure with a possible turn. There was a strongly hydrophilic region (residues 438–495). Toward the C terminus of this region, there was a highly charged segment predicted to be an α -helix (residues 470–492) and containing a large number of both positively and negatively charged residues, especially glutamic acid [61]. Elsewhere, it was reported that there were 37 % hydrophobic residues and 28 % polar residues [60]. Protein 4.2 did not show any obvious repeating primary structure, but a globular protein was suggested [60]. There were no extended stretches of β -sheet or α -helix. Instead, the protein was characterized by short segments. A hydropathy plot of protein 4.2 showed short alternating regions of hydrophobic and hydrophilic character [60]. The region of protein between amino acids 265 and 475, however, was characterized by two sets of alternating, prominent hydrophobic and hydrophilic domains [60].

6.2.4.3 Protein 4.2 Gene in Mouse Red Cells

There are substantial discrepancies between the three reports published on the protein 4.2 gene in mouse red cells [65–67]. Korsgren and Cohen (1994) described isolation of a 3.5 kb mouse P4.2 cDNA with the P4.2 transcript of 4.1 kb from mouse reticulocytes [65]. Rybicki et al. (1994), on the other hand, reported isolation of a full-length P4.2 cDNA of 2.2 kb from mouse reticulocytes [66]. Karacay et al. (1995) described an entire P4.2 cDNA sequence consisting of 3465 nt with an open reading frame (ORF) of 691 amino acids, and despite its similarity to human P4.2 cDNA, the mouse cDNA had a longer 3' untranslated region [67]. In addition, they reported that the mouse reticulocyte P4.2 RNA did not exhibit alternative splicing in the region identified in human P4.2 RNA. The P4.2 gene in mice was mapped to murine chromosome 2 [68], in contrast to 15q15–q21 in the human red cell P4.2 gene.

6.2.4.4 Tissue-Specific Expression of the Mouse Protein 4.2 Gene and the Pallid Mutation

Immunoreactive forms of P4.2 with a molecular weight of 72 kDa, in addition to those larger or smaller than 72 kDa, have been detected in nonerythroid cells and tissues [40, 69–71]. Immunologic cross-reactivity between the red cell P4.2 protein and other cellular proteins has also been reported [40, 69–71].

Zhu et al. (1998) recently reported that expression of the mouse P4.2 gene was temporally regulated during embryogenesis and that the P4.2 mRNA expression pattern matched the timing of erythropoietic activity in hematopoietic organs [70]. It should be noted that, contrary to previous reports, P4.2 expression was de-

tected only in the erythroid cell-producing organs and circulating red cells during mouse embryonic development and in adult mice. They first analyzed poly A⁺ RNAs from various adult mice tissues by Northern blot analysis using a 714 bp mouse P4.2 cDNA fragment containing the 3' protein of the P4.2-coding region as the probe. A single 3.5 kb P4.2 transcript was detected at a relatively high level in the spleen, while little or no P4.2 hybridization was seen in other tissues examined (brain, lung, liver, skeletal muscle, kidney, or testis). They extended the use of mice embryos for their P4.2 expression studies. A P4.2 hybridization signal was first detected not at E6.5 days, but instead in primitive erythroid cells in E7.5 embryos. In E10.5 embryos, the P4.2 hybridization signal was detected only in the heart and blood vessels. In E12.5 embryos, there was a switch in the hematopoietic production sites from the yolk sac to the fetal liver. In E 16.5 embryos, the signal was greatly reduced in the liver, and was almost undetectable after birth. Finally, P4.2 gene expression became confined to the red pulp on postnatal day 7. No P4.2 specific labeling was observed in the white pulp, which consisted of germinal centers for lymphocytes, plasma cells, and macrophages. No P4.2 hybridization signal was detected in megakaryocytes. Therefore, the P4.2 message was specifically expressed in cells of erythroid lineage in postnatal hematopoietic organs [70].

The chromosomal location of the mouse P4.2 gene was near a mouse pallid (*pa*) mutation [71]. Pallid was found in a mouse with dilution of coat color, increased bleeding time, and abnormal lysosomal enzyme secretion, as a model of the platelet storage pool disease [72, 73]. Therefore, it has been suggested that the P4.2 gene may be related to the pallid mutation gene, and it has even been proposed that the P4.2 gene itself should be nominated as a "pallidin" [40, 65, 71]. Patients with P4.2 deficiency, however, do not have the platelet storage pool deficiency seen in *pa/pa* mice, and the mutant mice do not exhibit the hemolysis and spherocytosis observed in the P4.2 deficiency [41–43]. It has recently been shown that the P4.2 gene is distinct from the *pa* gene, and that changes in P4.2 in pallid mice were not responsible for the pallid mutation [68]. There have also been reports of P4.2 transcripts, in addition to spleen, in other tissues (kidney, heart, brain, and liver), and even in other cells (HeLa cells or HT-29 cells). As with the results for the immunoreactive forms of P4.2 previously detected in nonerythroid tissues or cells, neither P4.2 message nor protein 4.2 have been found in nonerythroid tissues and cells [70].

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7

Membrane Morphogenesis in Erythroid Cells

7.1

Introduction

The genome exists basically in the nucleus, and its expression is controlled by many factors including promoter functions and epigenetic control mechanisms; methylation, phosphorylation, acetylation, and chromatin packaging.

In general, the genomic messages expressed in the nucleus are transferred via mRNA into the Golgi apparatus in the cytoplasm to produce the determined proteins. The proteins produced in the cytoplasm should be carried by presently unknown transport proteins to the cell membranes. The proteins transferred near the cell membranes should be incorporated precisely into the determined positions of the stereotactic ultrastructure of the cell membranes. The proteins, which are placed in order in the cell membranes, are only allowed to express their cellular functions.

As regards the processes from genotypes to phenotypes, the genomic mutations of the determined red cell membrane protein genes and the protein contents in the cell membranes have actually been studied [1–5]. However, only some stages in these steps have been clarified. Postgenomic investigations will be critical to the elucidation of the formation of red cell membrane ultrastructure in the future.

The exact mechanisms of membrane morphogenesis and of the formation of red cell shape have still not been clarified. This problem has proved to be a significant stumbling block to the elucidation of the pathogenesis of red cell membrane disorders, especially hereditary spherocytosis, hereditary elliptocytosis and hereditary stomatocytosis, in which abnormal red cell shapes, such as those in microspherocytosis, elliptocytosis and stomatocytosis, are hallmarks of these disorders. In human beings, the role of the spleen, which determines the survival of red cells, normal and abnormal, should be critically considered. The process is totally independent of genomic states. Therefore, genotype is obviously only one of the definite determinants for phenotypic expressions.

Despite this overview of the background on red cell membrane disorders, elucidation of the mechanism of biomorphogenesis of the red cell membrane structure is still critically important to the understanding of the disease states of these disorders.

Studies of the morphogenesis of red cell membranes were initiated in the early 1980s utilizing non-human erythroid cells, in particular avian and murine erythroblasts [6–20]. It was found that glycophorins and spectrin were expressed in human proerythroblasts prior to the expression of band 3 [21, 22]. This observation was confirmed by us in cultured erythroid cells, which were obtained from a burst-forming unit in erythroid (BFU-E) in peripheral blood by the two-phase liquid culture method [23, 24] (Figs. 7.1 and 7.2, and Table 7.1). In our study, protein 4.1 appeared in intermediary matured erythroblasts on days 7–9 of the second phase of this method [24]. Protein 4.2 was expressed only in the erythroblasts at the latest stage (after day 10), which was equivalent to the stages from orthochromatic erythroblasts to reticulocytes [24]. The mRNA for protein 4.2 was first expressed on day 3 of the second phase [24]. These findings showed clearly that the red cell membrane proteins were expressed sequentially during erythroid maturation: i. e., firstly spectrin and band 3, then protein 4.1 and finally protein 4.2. These were then incorporated into the erythroid membranes sequentially [24]. The presence of several gene products, which corresponded to the intermediate isoforms of protein 4.2 protein, was also observed on protein 4.2 mRNA [24]. The gene products consisted of seven isoforms of different sizes, which were produced by various combinations of exon skipplings. In disease states, several isoforms of protein 4.2 are known to be present in human erythroid cells: i. e., 72, 74, 67 kDa and others [25]. The appearance of these protein 4.2 isoforms has been suggested to be closely linked with the gene control mechanism in erythroid morphogenesis, and also with posttranslational modification, typically shown in the protein 4.2 doublet Nagano [26].

Table 7.1 Morphological profile of cultured cells during second phase of the two-phase liquid culture method.

	<i>Percentage of cells (% , mean $\pm$ SD)</i>			
	<i>Cultured cells in the second phase of two-phase liquid culture method</i>			
	<i>Day 0</i>	<i>Day 5</i>	<i>Day 9</i>	<i>Day 16</i>
Blastic cells/lymphoid cells	98.2 $\pm$ 0.3	54.5 $\pm$ 5.3	9.5 $\pm$ 1.4	7.2 $\pm$ 1.5
Nucleated cells				
Immature erythroblasts*	1.2 $\pm$ 0.1	39.2 $\pm$ 4.3	22.2 $\pm$ 2.3	4.1 $\pm$ 1.8
Mature erythroblasts**	0	5.1 $\pm$ 0.7	50.3 $\pm$ 2.4	60.3 $\pm$ 3.2
Enucleated erythroid cells	0	0.3 $\pm$ 0.1	17.2 $\pm$ 1.3	27.2 $\pm$ 2.9
Myeloid cells	0.6 $\pm$ 0.1	0.9 $\pm$ 0.2	0.8 $\pm$ 0.1	1.2 $\pm$ 0.3

* Equivalent to proerythroblasts, basophilic erythroblasts and polychromatic erythroblasts.

** Equivalent to orthochromatic erythroblasts.

Differential counts were performed on 400 cells in duplicate each time in ten independent experiments.

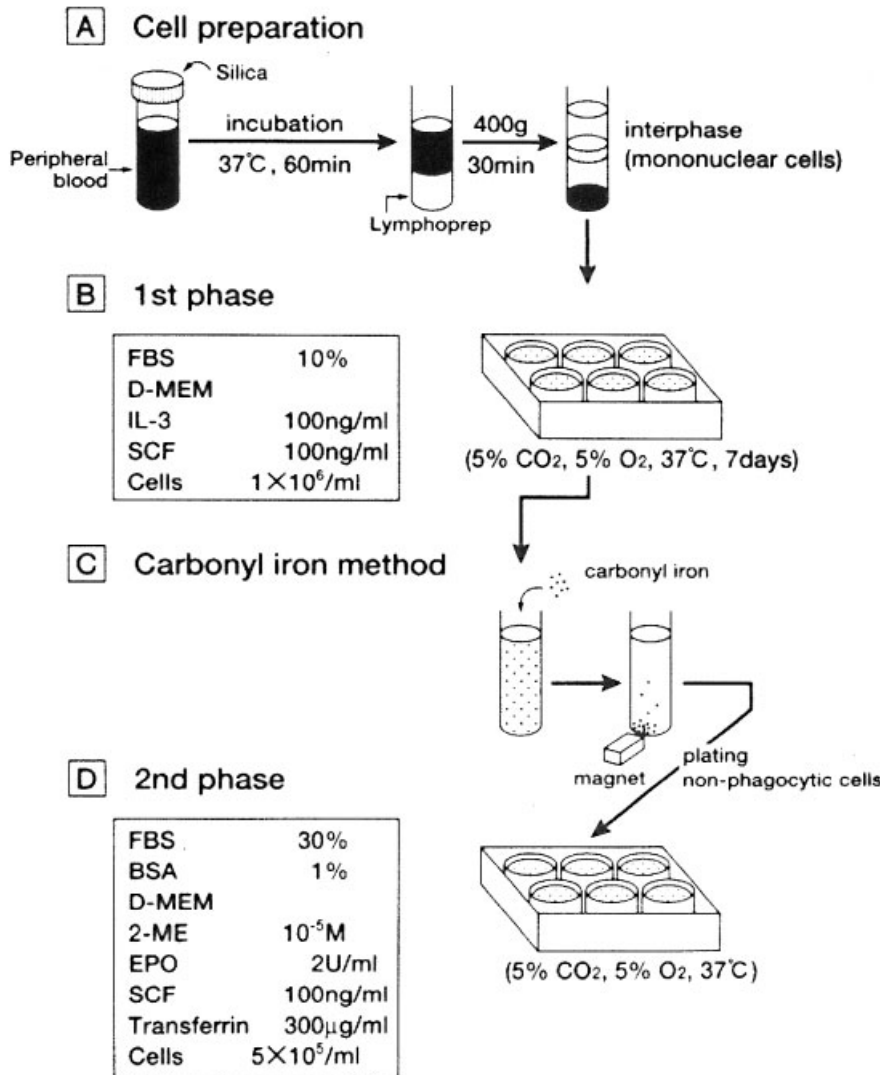


Figure 7.1 Two-phase liquid culture method for studying human erythroid cells. [A] Normal peripheral mononuclear cells (1×10^7) were obtained from 30 mL of peripheral blood by utilizing Lymphoprep. [B] The mononuclear cells (1×10^6 cells per mL) were then incubated with an Iscove's modified Dulbecco's medium (D-MEM) containing 100 ng mL⁻¹ recombinant human interleukin 3 (Kirin Brewery Co., Tokyo, Japan), 100 ng mL⁻¹ recombinant human stem cell factor (SCF: Kirin Brewery Co., Tokyo, Japan), and 10% fetal bovine serum at 37°C for 7 days under 5% CO₂ and 5% O₂ (the first phase). [C] After the incubation, phagocytic cells were removed by the addition of carbonyl iron. [D] Nonphagocytic cells (5×10^5 cells per mL) were further incubated with 30% fetal bovine serum, 1% bovine serum albumin, 1×10^{-5} M 2-mercaptoethanol, 2 units per mL of erythropoietin (Kirin Brewery Co.), 100 ng mL⁻¹ of SCF, 300 μg mL⁻¹ of iron-saturated transferrin, and D-MEM at 37°C under 5% CO₂ and 5% O₂ (the second phase). On day 9, 2 units per mL of fresh erythropoietin were added to the cell suspension. Further incubation was carried out to day 16.

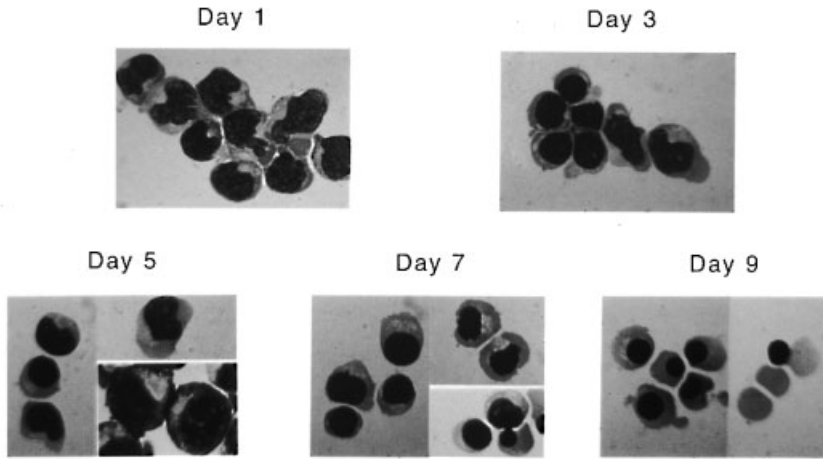


Figure 7.2 Morphology of the cultured cells corresponding to days 1, 3, 5, 7, and 9 of the second phase of the two-phase liquid culture method. Representative results with Wright–Giemsa stain are shown.

7.2

Red Cell Membrane Proteins During Erythroid Development and Differentiation

Red cell membrane proteins are functionally classified into three groups [1–5]. The first one is a group of proteins which lie in parallel just beneath the lipid bilayer. These proteins form a cytoskeletal network as a complex of α -spectrin, β -spectrin, protein 4.1, actin, and other minor proteins through horizontal interactions (Fig. 2.2). The second group is a group of proteins which exist in the lipid bilayer. These integral proteins are band 3, glycophorins, and other minor proteins. A complex of band 3– β -spectrin–ankyrin, and a complex of spectrin–protein 4.1–glycophorin are identified by their vertical interactions (Fig. 2.2). The third group is a group of proteins which connect skeletal proteins to integral proteins. These anchoring proteins are protein 4.2, ankyrin, p55, and other minor proteins. They form complexes of glycophorin C–p55–protein 4.1–spectrin, of band 3–protein 4.2, and of protein 4.2–spectrin.

The interactions between these membrane proteins are critical for maintaining the normal integrity of the red cell membrane ultrastructure [1–5]. They are synthesized in the cytoplasm of erythroid cells and incorporated into the erythroid membrane structure during erythroid development and differentiation.

7.2.1

Expression of Membrane Proteins in Early Erythroid Progenitors (BFU-E and CFU-E)

The expression of membrane skeletal proteins has been studied usually by utilizing burst-forming units in erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) in avian cells transformed by the erythroblastosis virus (AEV) or S13

virus [6–11], in murine erythroleukemia cells (MEL) [12, 13], or in mouse progenitor cells transformed by Friend virus (FVA) [14–16] or Rauscher erythroleukemia virus [17]. These cells can differentiate into erythroid cells in the presence of erythropoietin and DMSO. The basic mechanism for expression of these membrane proteins appears to be similar in avian cells [6–11, 20] and mammalian cells [12–19]. The most substantial studies in this field were carried out in avian cells by Lazarides and his group.

The expression of β -spectrin starts with the appearance of γ -spectrin, which is considered as a precursor form of β -spectrin [6]. During erythroid maturation at the stage of the postmitotic phase, γ -spectrin is switched into β -spectrin [6]. At the stage of erythroid progenitors, α -spectrin, β -spectrin, protein 4.1 and ankyrin expression has been detected, but at a minimal level [9, 14, 17]. These newly synthesized proteins are present mostly in the cytoplasm and are incorporated only minimally into the membranes. Hanspal et al. [17] demonstrated that only a small (4–8) percentage of newly-synthesized proteins can be assembled into the membranes at the CFU-E stage. However, when these cells were induced to early erythroblasts, fractions of the newly synthesized proteins, which were assembled into the membranes, increased up to 12.8–23.5% [17]. Lazarides et al. [1, 9–11] reported that the structure of the cytoskeletal network at the stage of erythroid progenitors is extremely unstable, because the high-affinity binding domain for ankyrin–spectrin–protein 4.1 is lacking, and also because band 3 and glycophorin C are not expressed on the membranes at this stage [9–11]. Although spectrin dimers or tetramers, and in some situations, even β_4 - or α_2 -spectrin chains, are formed, these complexes are easily catabolized due to their molecular instability [9, 10] (Fig. 7.3).

7.2.2

Expression of Membrane Proteins in Early Erythroblasts

At this stage, band 3 protein is expressed, and thereby both vertical interactions (the band 3–ankyrin–spectrin complex, and the glycophorin C–protein 4.1–spectrin complex) and horizontal interactions (spectrin–protein 4.1–actin) are established [9, 10, 12, 14, 16, 17, 21]. These are membrane structures specific for erythroid cells [21].

Lazarides et al. proposed the following mechanism for the assembly of the membrane structure based on their studies on avian cells [10]. Amongst each membrane protein, there are high affinity binding sites [27, 28]. Once the membrane proteins are synthesized, they are subjected to conformational changes by chemical modifications through phosphorylation, lipid acylation, and others. In addition, the expression of membrane proteins is not synchronized [1, 9, 10, 13, 14, 16, 17, 19, 21]. The ankyrin–spectrin–protein 4.1 complex appears to be primarily produced as a horizontal interaction [10, 11]. Then vertical interaction by the band 3–ankyrin–spectrin complex or the glycophorin C–protein 4.1–spectrin complex follows [10, 11]. These interactions of membrane proteins are regulated precisely by phosphorylation, and various posttranslational modifications [10, 11].

A

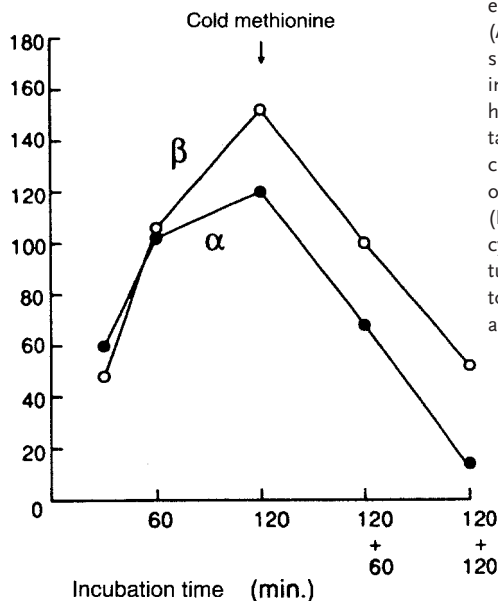
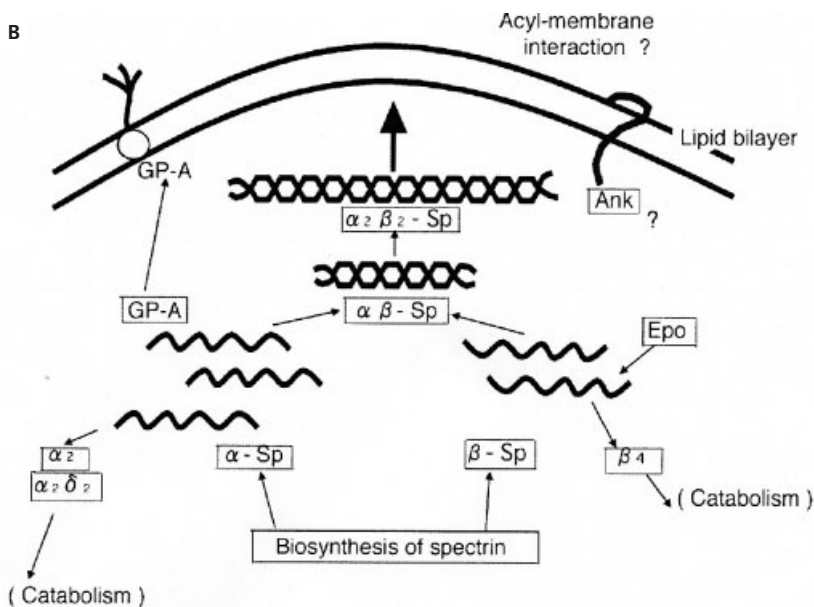
Incorporation of ^{35}S -methionine into spectrins


Figure 7.3 Membrane biogenesis in erythroid progenitors. (A) Enhanced synthesis of β -spectrin (β) over α -spectrin (α) in the cytoplasmic fraction of human early erythroblasts obtained by the two phase liquid culture method in the presence of erythropoietin (2 units per mL). (B) Transient assembly of the cytoskeletal complex and its turnover in erythroid progenitors. GPA: glycophorin A, Ank: ankyrin, Sp: spectrin.

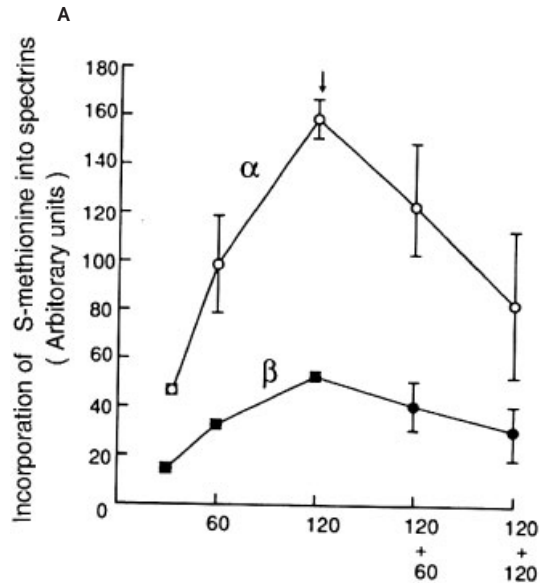
B



Palek et al. confirmed the above-mentioned concept by basing their results on human erythroid cells, and proposed the presence of three rate-limiting steps [17, 21]. In the first one, α -spectrin is synthesized in a three times greater excess than β -spectrin (Fig. 7.4A). Therefore, β -spectrin is definitely rate-limiting for the formation of the spectrin dimer [7, 21]. In the second step, the spectrin dimer is bound to ankyrin tightly and specifically [27]. In the third step, the ankyrin molecule, which is bound to a spectrin dimer, binds further to band 3 specifically [28]. These two proposals made by Lazarides [10] and Palek et al. [17, 21] fit well with results observed in mouse models of hereditary spherocytosis [19] and human patients with spectrin deficiencies [22]. The observations made in these particular mouse models are as follows. (1) When the expression of β -spectrin is reduced or totally deficient and the expression of α -spectrin is maintained normally [22], α -spectrin and β -spectrin contents are equally diminished. This clearly indicates that the formation of spectrin dimers by α -spectrin and β -spectrin is critically important. The expression of spectrins *per se* does not affect the expression of other membrane proteins, such as band 3 [11, 21]. (2) When the expression of ankyrin is partly deficient, the contents of α -spectrin and β -spectrin are diminished in addition to a partial ankyrin deficiency in mature red cells [13]. At such a time, the expression of band 3 is unaffected, and band 3 is present normally [1, 21]. (3) In deficiencies of protein 4.1 or glycophorin C, the contents of spectrin, ankyrin, and band 3 are maintained normally [29], indicating that an abnormality in the glycophorin C–protein 4.1–spectrin complex associated with the vertical interaction does not appear to affect the formation of the cytoskeletal network [10, 11, 21].

Figure 7.4 Membrane biogenesis in early erythroblasts.

(A) Predominant biosynthesis of α -spectrin (α) over β -spectrin (β), and their catabolism in the cytosolic fraction of human early erythroid cells obtained by the two phase liquid culture method. (B) Incorporation of α - and β -spectrins into the membrane fraction equally in human erythroid cells obtained by the two phase liquid culture method. EPO: erythropoietin.



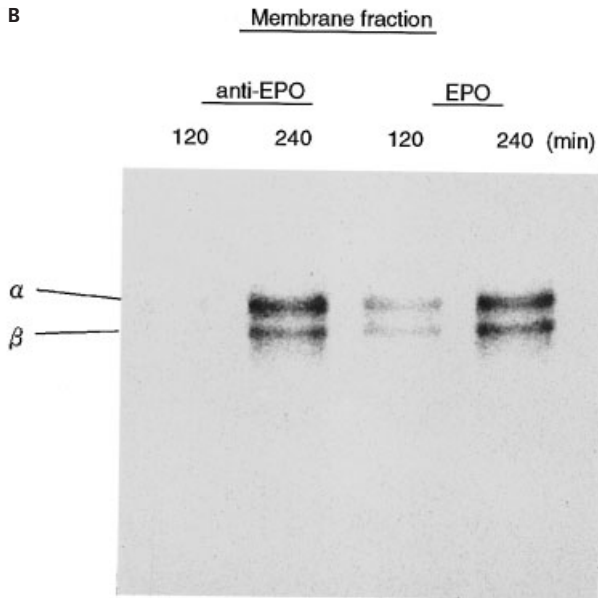


Figure 7.4 Part B.

Erythropoietin (EPO) is known to induce the differentiation of CFU-E into erythroblasts (Fig. 7.4 B). Hanspal et al. [15] demonstrated that, in the presence of erythropoietin, an incremental synthesis of spectrin in the soluble fraction is observed only in β -spectrin, but not in α -spectrin. The increased β -spectrin content is due to increased mRNA content. Furthermore, in the presence of erythropoietin, the net incorporation of spectrins is markedly increased in parallel with α -spectrin and β -spectrin. Thus, β -spectrin is definitely a rate-limiting factor in both the synthesis of spectrins and in their assembly into the membrane structure [7, 15].

7.2.3

Expression of Membrane Proteins in Late Erythroblasts

At the stage of late erythroblasts during erythroid development and maturation, the basic structure of the erythroid membrane appears to be almost completed [10, 21]. However, it may still be unstable and fragile. It can easily be imagined that some other membrane proteins may be required to biophysically and functionally strengthen the interactions between the cytoskeletal network and the membrane lipid bilayer. Assembly of these membrane proteins into the membrane structure may be crucial to finalizing the construction of the normal ultrastructure.

It has been calculated that total membrane protein content in late erythroblasts is approximately 80% of that in mature red cells [21]. The membrane proteins incorporated into the membranes in late erythroblasts are stable with a lower turnover rate than in early erythroblasts. In late erythroblasts, the amounts of spectrin,

ankyrin, and band 3 synthesized are clearly reduced compared with those in early erythroblasts [16]. Contrary to the reduced synthesis of these membrane proteins, the biosynthetic rate of protein 4.1 is increased in late erythroblasts. Protein 4.1 is considered to fortify the cytoskeletal network.

Since the assembly of protein 4.2 has not been well elucidated, we investigated this aspect in human erythroblasts from bone marrow and in erythroid-committed cultured cells obtained from BFU-E in peripheral blood by the two phase liquid culture method, as described in detail in the next section (Section 7.3). Protein 4.2 was detected in 9% of the erythroblasts that expressed band 3, suggesting that band 3 is clearly expressed prior to protein 4.2 [24]. The expression of protein 4.2 was observed after the expression of protein 4.1 [24]. Thus, protein 4.2 appears to be the membrane protein which is expressed last amongst the various major membrane proteins during erythroid development and differentiation. This protein appears to be expressed in orthochromatic erythroblasts just before enucleation, through which reticulocytes are produced [24]. Therefore, protein 4.2 may play some role in this enucleation event, although the detailed functions of protein 4.2 have not yet been clarified. The functional targets of protein 4.2 appear to be band 3 and spectrin, suggesting that protein 4.2 may strengthen the vertical and horizontal interactions after the membrane ultrastructure is virtually completed, or regulate these assemblies at the last moment during the maturation of erythroid cells [25] (Fig. 7.5).

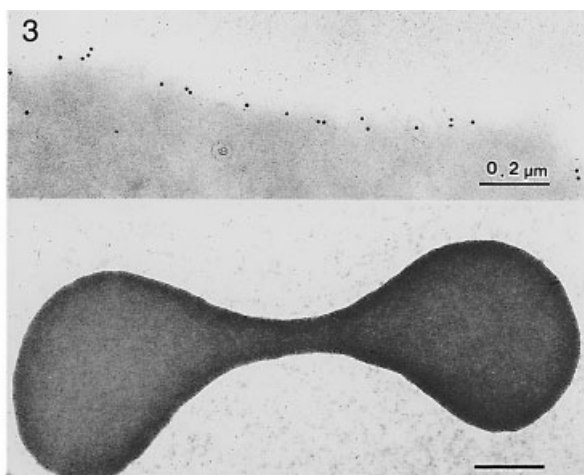
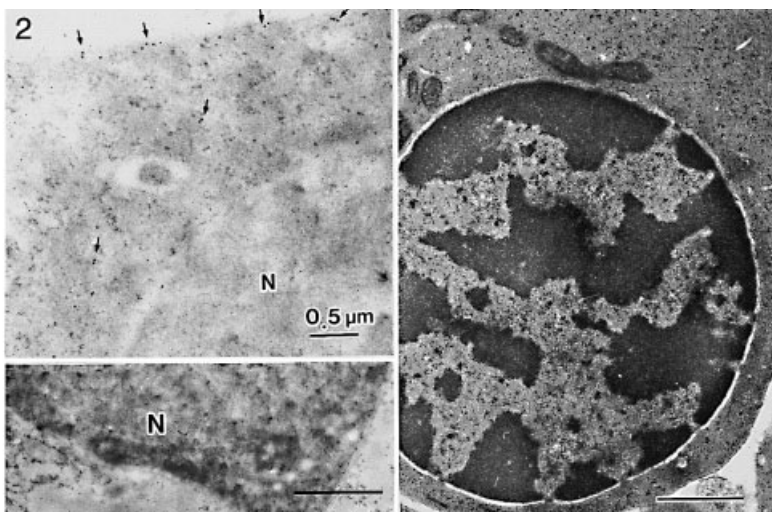
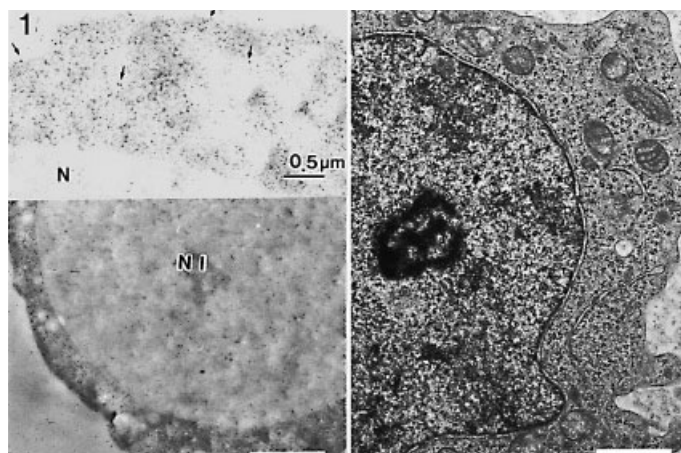
Figure 7.5 see page 142–144

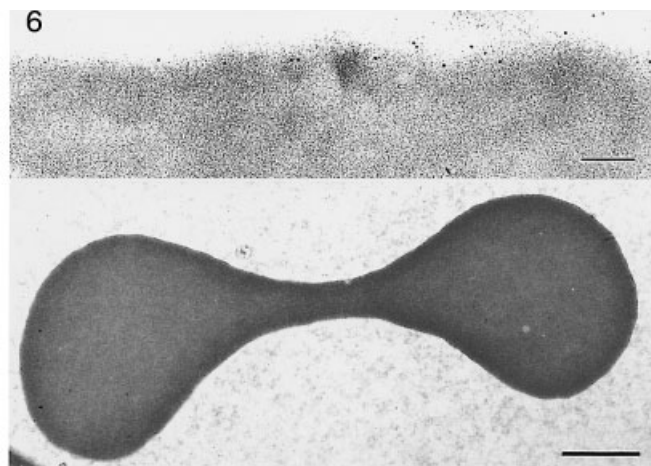
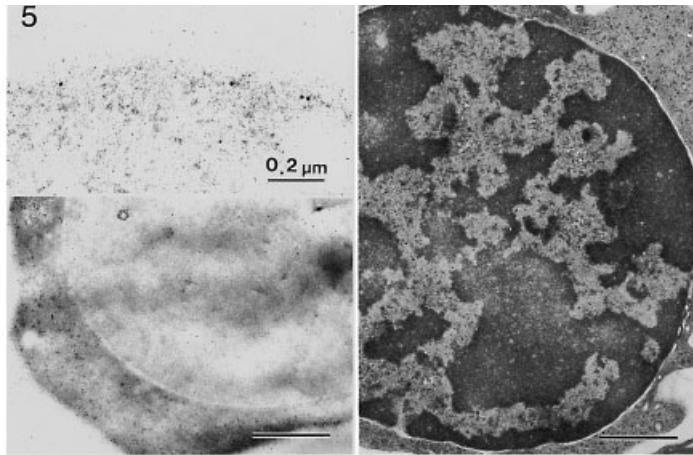
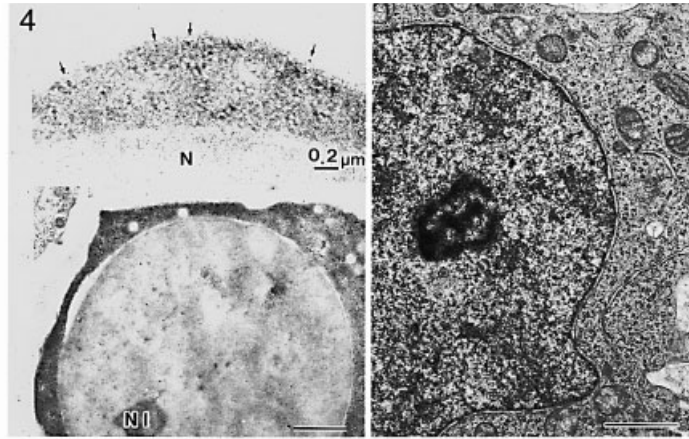
Figure 7.5 Expression and development of membrane proteins in erythroid cells *in vivo* of bone marrow detected by the immunogold method.

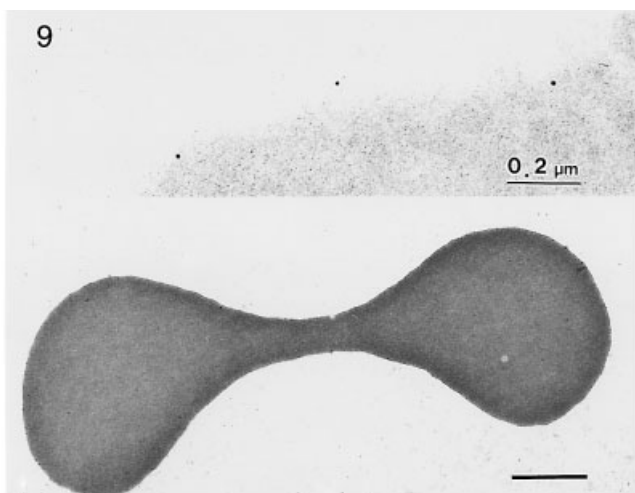
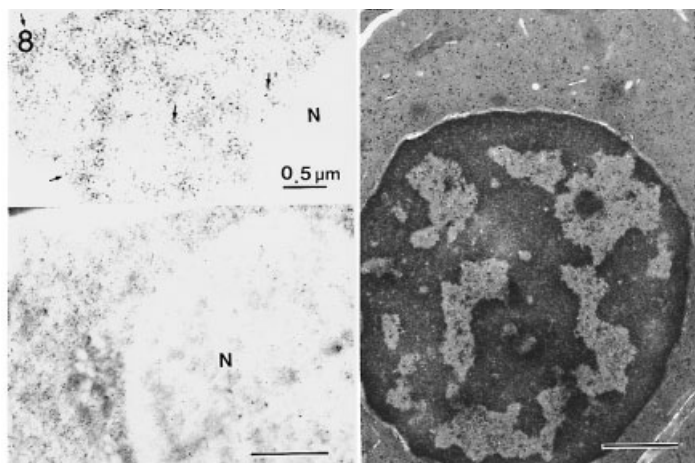
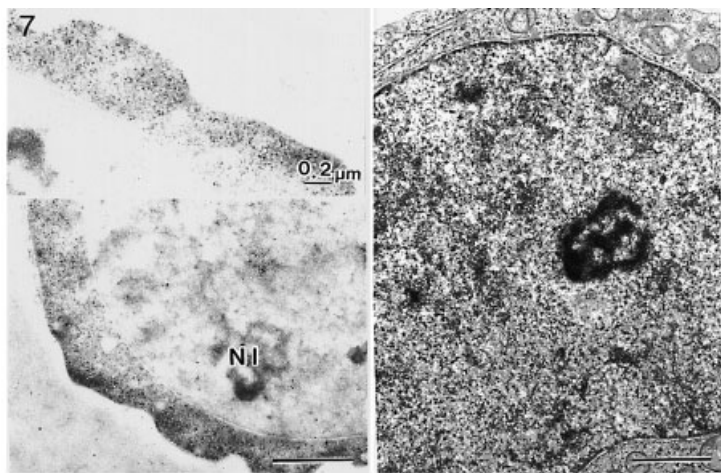
- 1, Spectrins in proerythroblasts.
- 2, Spectrins in late erythroblasts.
- 3, Spectrins in red cells.

- 4, Band 3 in proerythroblasts.
- 5, Band 3 in late erythroblasts.
- 6, Band 3 in red cells.
- 7, Protein 4.2 in proerythroblasts.
- 8, Protein 4.2 in late erythroblasts.
- 9, Protein 4.2 in red cells.
- N: nucleus, NI: nucleolus.

In each set, electron micrographs by regular transmission electron microscopy (right) and by immuno-electron microscopy with antibodies against spectrins, band 3, or protein 4.2 (upper left) and without them (lower left) are shown. Arrows indicate immunogold particles.







7.3

Sequential Expression of Erythroid Membrane Proteins, Particularly Protein 4.2

7.3.1

Expression of Red Cell Membrane Protein 4.2 and Its mRNA in Normal Human Erythroid Maturation

The expression of membrane proteins in erythroid cells has been studied in various species, i.e.: in avian cells transformed by the avian erythroblastosis virus (AEV) or S-13, in murine erythroleukemia cells (MEL), and in erythroid progenitors transformed by the Friend virus (FVA) or Rauscher erythroleukemia virus, which can differentiate into mature erythroblasts. It has become clear that spectrin as a cytoskeletal protein has already been expressed in early erythroid progenitors, followed by sequential expression of band 3 as an integral protein in early erythroblasts. In contrast, there has been little precise description of the expression of protein 4.2 during erythroid maturation, although indirect evidence has been found in the peripheral red cells of a mouse given a single injection of ^{35}S -methionine, by which protein 4.1 and 4.2 appeared to be synthesized at the same level throughout erythropoiesis [18].

In a study using the two phase liquid culture method, in which normal peripheral mononuclear cells containing the burst-forming unit-erythroid (BFU-E) were utilized, sequential expression of membrane proteins during the maturation of human erythroid precursors has recently been shown [24]. Using this method, spectrin was detected at the early stage of the second phase (probably earlier than proerythroblasts), followed by band 3 in early erythroid cells (Fig. 7.6). Protein 4.1 was detected on day 9 of the second phase of this method, corresponding to protein 4.1b, while peripheral blood cells exhibited both protein 4.1a and 4.1b (Fig. 7.6). Protein 4.2, however, was barely recognized even after so many days of culture. It first appeared on day 10, when spectrin and band 3 were definitely expressed. The content of protein 4.2 in the cultured erythroid cells on day 10 was approximately $5 \pm 4\%$ of that in the same number of peripheral red cells (Fig. 7.6). The extent of protein 4.2 expression increased sequentially, to $29 \pm 10\%$ on day 14, and $37 \pm 18\%$ on day 16, when, in addition to a major band (72 kDa) of protein 4.2, two more faint bands were detected, by Western blotting with antiprotein 4.2 antibody, probably as isoforms of protein 4.2 (Fig. 7.7). Flow cytometric analyses confirmed these results.

At the mRNA level, no protein 4.2 mRNA was detected in normal cultured erythroid cells even by RT-PCR on day 0 of the first and second phases of this culture method [24]. After day 3, protein 4.2 mRNA was detected by RT-PCR. Using Southern blot analysis, seven bands of protein 4.2 RT-PCR products were detected on days 3 and 8 of the second phase by autoradiography. These seven bands were also detected in reticulocytes in peripheral blood, but there were none in mononuclear cells present in peripheral blood. The sizes of the detected products were (I) 1052, (II) 986, (III) 915, (IV) 889, (V) 813, (VI) 709, and (VII) 595 base pairs, which appeared to correspond to the theoretical sizes deduced from the protein 4.2 se-

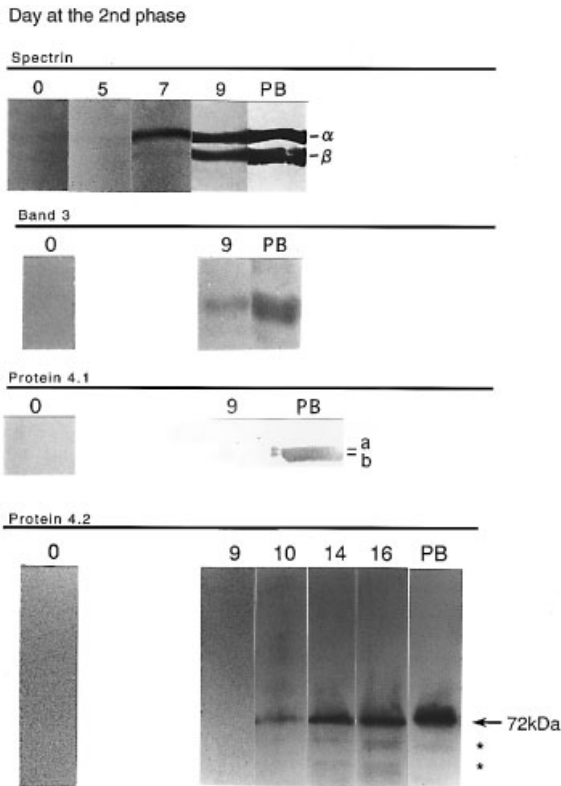


Figure 7.6 Expression of spectrin, band 3, protein 4.1, and protein 4.2 during maturation of erythroid cells by the two phase liquid culture method. The whole lysates of erythroid cells cultured by the two phase liquid culture method were subjected to regular Western blotting with anti-human antibodies on SDS–PAGE. By day 9 of the second phase in the culture method, α -spectrin, β -spectrin, band 3, and protein 4.1

were detected. This protein 4.1 corresponded to protein 4.1b, while peripheral blood cells (PB) exhibited both protein 4.1a and 4.1b. On this day, protein 4.2 had still not been expressed. Protein 4.2 was first detected on day 10, and gradually increased in amount. In protein 4.2, a major 72 kDa peptide as a wild form and two minor bands (63 and 59 kDa) in trace amounts were also detected as shown by asterisks (*).

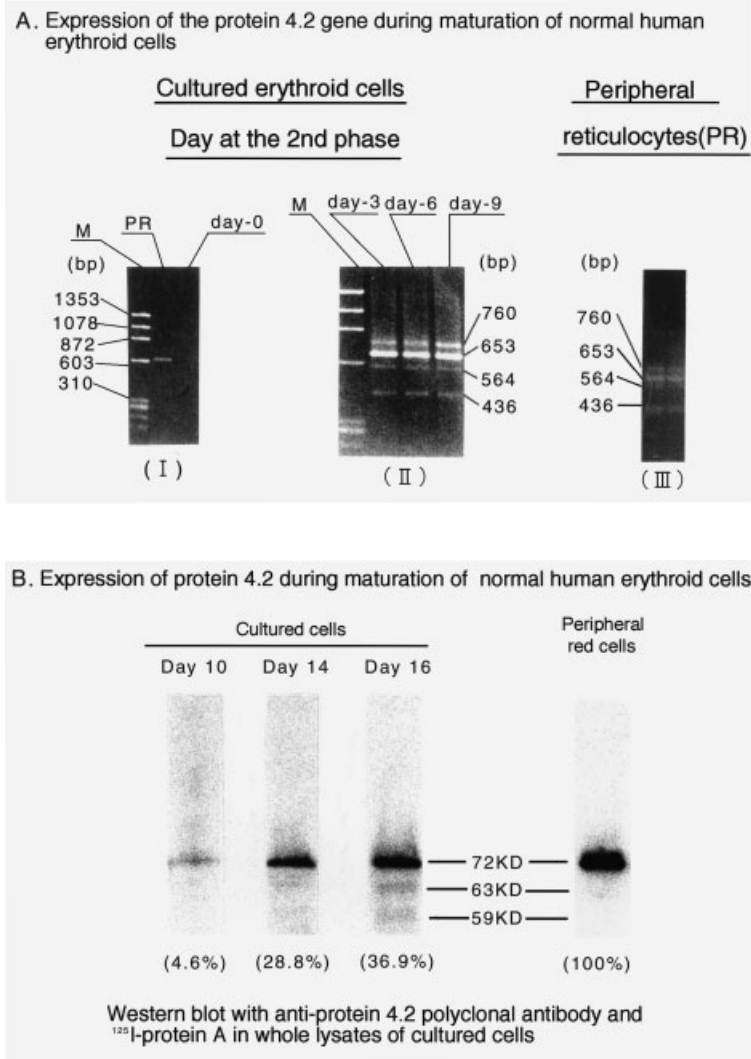


Figure 7.7 Expression of protein 4.2 and its gene during maturation of normal human erythroid cells. A, Expression of the protein 4.2 gene in cultured cells by the two phase liquid culture method and in peripheral reticulocytes.

B, Expression of protein 4.2 during maturation of normal human erythroid cells detected by the Western blotting with anti-human protein 4.2 polyclonal antibodies.

quence, that is, 1070, 980, 965, 875, 836, 746 or 712, and 641 base pairs, respectively. These findings were also confirmed by silver staining. At the cDNA level, these products correspond to (TypeI) 2163, (II) 2073, (III) 2058, (IV) 1968, (V) 1929, (VI) 1839, (VI') 1824, and (VII) 1734 base pairs, which are isoforms deduced directly by gene sequences (Fig. 7.8). The largest isoforms (TypeI) of protein 4.2 were produced by reading the full length of the protein 4.2 gene. A wild type of the 72 kDa (II) was obtained by the skipping of 90 nucleotides in exon 1. Through sequencing, other isoforms were also identified and obtained: by skipping only exon 5 (III); by combined skipping of 90 nucleotides in exon 1 and exon 5 (IV); by skipping exon 3 (V); by combined skipping of 90 nucleotides in exon 1 and

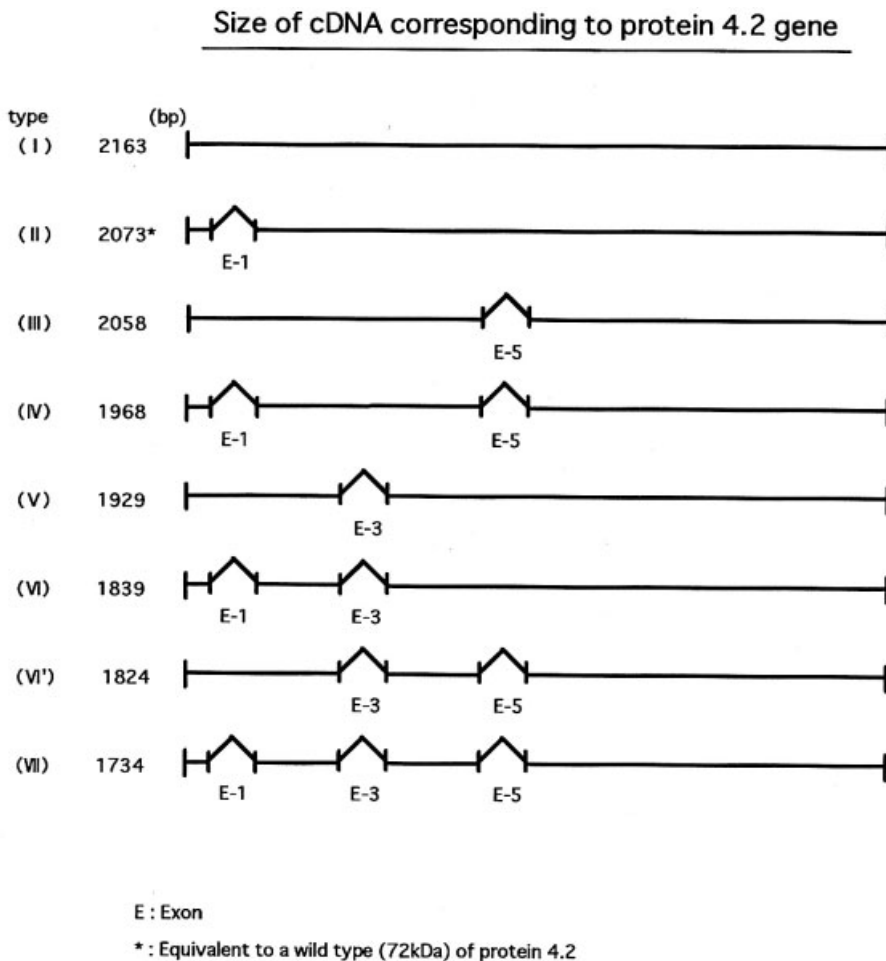


Figure 7.8 Presence of seven products of human erythroid protein 4.2 pre-mRNA or mature RNA produced by various exon skipplings. The expression of seven gene products among them, however, were confirmed by Southern blotting.

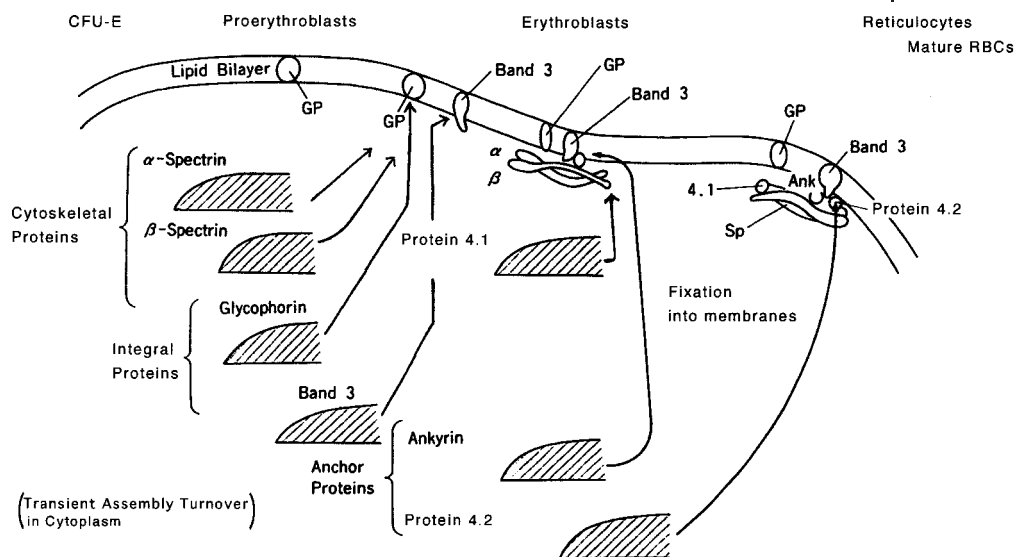


Figure 7.9 Overall view of sequential expression of membrane proteins in human erythroid cells during their maturation. CFU-E: colony-forming unit in erythroid, GP: glycophorin, RBC: red cells.

exon 3 (VI); by skipping exon 3 and 5 (VI'); and by combined skipping of 90 nucleotides in exon 1, exon 3, and exon 5 (VII) [24] (Fig. 7.8).

These findings clearly indicate that, in sequence, spectrin was expressed in very early erythroblasts, followed by band 3 and protein 4.1. Then protein 4.2 was expressed in very late erythroblasts or around the stage of the reticulocytes. Thus, the expression of protein 4.2 appears to be the last event amongst the various membrane proteins [24] (Fig. 7.9).

In disease states, several isoforms of protein 4.2 are known to be present in human erythroid cells, i.e.: 72 kDa, 74 kDa, 67 kDa, etc. [25]. Even in normal red cells, at least three isoforms of protein 4.2 have been detected by Western blotting with the Protein-A method [24, 25]. The significance of the appearance of these isoforms of protein 4.2 is unknown. The 72 kDa and 74 kDa peptides were detected in trace amounts by Western blotting in the red cells of protein 4.2 deficiency of the Nippon type (a homozygote of codon 142 GCT→ACT) [30], and the 74 kDa peptide in heterozygotes of protein 4.2 Shiga (codon 317 CGC→TGC) [31] and heterozygotes of protein 4.2 Komatsu (codon 175 GAT→TAT) [32]. Variants of protein 4.2, in which 72 and 74 kDa peptides were equally or nearly equally expressed in red cells, have also been reported as a protein 4.2 doublet Kobe and a protein 4.2 doublet Nagano [26]. Therefore, the control mechanism of expression of protein 4.2 should be clarified with respect to the phenotypic expression of protein 4.2.

7.3.2

Developmental Expression of Mouse Red Cell Protein 4.2 mRNA in Erythroid and Nonerythroid Tissues

It has been shown that erythropoiesis in mouse embryos is initiated at the yolk sac (E7.5–11.5 days) as a primitive erythropoiesis, by which nucleated erythroblasts are produced from the blood islets. At E12.5–16.5 days, the liver becomes the major erythropoietic organ producing erythroblasts, which can be differentiated into enucleated red cells in the peripheral blood, as a definitive erythropoiesis. After E16.5 days, a reduction in the hematopoietic function of the hematopoietic cells in the liver begins and their proliferation decreases. Around and after birth, the spleen and the bone marrow become the major hematopoietic organs in mice [33].

Expression of the mouse protein 4.2 gene, the protein 4.2 mRNA, and protein 4.2 itself has recently been studied by Northern blot analysis and by *in situ* hybridization during mouse embryogenesis [33]. To assess expression of the protein 4.2 gene, poly A⁺ RNAs from various adult mouse tissues were analyzed by Northern blot analysis using a 714-base pair mouse protein 4.2 cDNA fragment containing the 3' portion of the protein 4.2-coding region as the probe. A single 3.5 kb protein 4.2 transcript was detected at a relatively high level in the spleen. Little or no protein 4.2 hybridization was observed in other tissues examined (brain, lung, liver, skeletal muscle, kidney, or testis) [33].

At different stages of development, no protein 4.2 hybridization was detected in mRNAs from E6.5 embryos. The expected 3.5 kb protein 4.2 mRNA was first detected in E7.5 embryos, and its intensity increased in the embryos at later ages (E14.5 and E16.5). Protein 4.2-specific labeling was found to be localized in primitive erythroid cells. Starting from E12.5 days, there was a switch in the hematopoietic sites from the yolk sac to the fetal liver, where protein 4.2 expression was observed. By E14.5 days, the liver showed the strongest protein 4.2 hybridization signal among all organs. The protein 4.2 hybridization signal was greatly reduced in the liver after E16.5 days, and was almost undetectable after birth, when the spleen and bone marrow became the major hematopoietic sites. Significant protein 4.2 expression was seen in the emerging red pulp of the spleen [33].

Throughout embryogenesis, no protein 4.2-specific labeling was observed in the lymphocytic organs (the thymus, the white pulp and germinal centers of the spleen), lymphocytes, plasma cells, macrophages, or megakaryocytes, but was detected in erythroid cells, and particularly at high intensity in areas with more mature erythroid cells [33]. Even in the bone marrow, no protein 4.2 hybridization signal was seen in cells of nonerythroid lineage, including megakaryocytes [33]. The protein 4.2 message was specifically expressed in cells of erythroid lineage in post-natal hematopoietic organs.

Immunoreactive forms of the red cell protein 4.2 have been reported in nonerythroid tissues such as brain, kidney, heart, liver, platelets, human endothelial cells, HT-29 human colon adenocarcinoma cells, T-84 human colon carcinoma cells, SK-N-MC human neuroblastoma cells, HeLa human cervical epithelial carcinoma

cells, a CV-1 monkey kidney fibroblast cells, and an Sf9 insect cell line [34–37]. The most recent work detected no protein 4.2 message in nonerythroid organs or megakaryocytes by Northern blot analysis [33]. Therefore, the immunoreactive forms of protein 4.2 detected in nonerythroid tissues or cells, which have been reported previously, do not appear to be protein 4.2 isoforms, although they may be protein 4.2-related proteins. Further characterization of the molecular nature of these protein 4.2-immunoreactive analogs is needed to identify them.

The protein 4.2 gene in mice is mapped to mouse chromosome 2 [38]. Its chromosomal location was found to be near the mouse pallid (*pa*) mutation [34, 35, 38]. Pallid is one of several independent mouse mutations that are models of the platelet storage pool disease [39, 40]. With Southern blot and Northern blot analyses, it has been suggested that this pallid mutation (*pa*) in mice is a mutation in the protein 4.2 gene [35, 36, 38]. Under the above-mentioned assumption, the name of “pallidin” has been proposed for protein 4.2 [34]. However, human patients with protein 4.2 deficiency have not shown any abnormalities of the platelets, such as the platelet storage disease, observed with the pallid mutation (*pa/pa*), but have demonstrated uncompensated hemolytic anemia [25, 30–32]. The mutant (*pa/pa*) mice did not have the increased hemolysis and spherocytosis seen in human patients [39, 40]. Recently, the proposal that pallid is a mutation in the protein 4.2 (*Epb 4.2*) gene has been weakened by the fact that the gene encoding protein 4.2 is distinct from the mouse platelet storage pool deficiency mutation pallid [41]. Therefore, the name “pallidin” is inappropriate and misleading in describing protein 4.2.

It also has been reported that the red cell protein 4.2 gene (*Epb 4.2*) was present in normal platelets and absent from protein 4.2-null mice produced by the targeted protein 4.2 gene, although normal platelet counts with normal platelet functions have been observed in protein 4.2-null mice [42]. In this study, the immunoblotting method was utilized to detect the presence of protein 4.2 in normal mice [42]. The discrepancy between this finding and those by other investigators [33] should be clarified in the near future.

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8

States of Methylation in the Promoter of the Genes of β -Spectrin, Band 3, and Protein 4.2

8.1

Introduction

Methylation patterns in human and eukaryotic genomes have often been investigated for their significance in determining activity profiles of eukaryotic genes. Specific modes of promoter sequence methylation can be linked to long-term gene inactivation [1]. In some instances, promoters completely methylated in all 5'-CG-3' dinucleotides retain full activity, such as promoters from frog virus (FV3) in fish or hamster cells [2]. One of these FV3 promoters responds with inactivation when merely the 5'-CCGG-3' sites are methylated. Specific patterns rather than the extent of promoter methylation are likely to be responsible for promoter inactivation. However, DNA methylation patterns in a genome can exert functions beyond those regulating promoter activity. Since a 5'-methyldeoxycytidine (5-mC) residue at a specific site in a nucleotide sequence can function as a positive or a negative modulator of protein–DNA interactions, the topological arrays of unique chromatin structures in different parts of a genome might depend on unique patterns of methylation as the primary regulators in the chain of protein–DNA interactions, on which chromatin structure is dependent.

Work on DNA methylation is still characterized by uncertainties of its biological functions. The highly conserved, in part inter-individually identical patterns of distribution of 5-mC residues, that is, in the human genome, point to a role transgressing that of the long-term inactivation of mammalian promoters [1, 3–8]. These patterns might provide a framework for chromatin scaffolding in that 5-mC residues modulate the primary direct interaction of DNA with the inner-most layer of proteins. The scaffold thus generated then establishes the imprint for a multitude of protein–protein interactions that contribute to the formation of the specific chromatin structures established in different parts of the mammalian genome. For this reason, our interest has focused on the determination of methylation patterns in mammalian, in particular human, genome segments [9].

During studies of the genotypes and phenotypes of red cell membrane disorders, it was observed that, among the two alleles present, one abnormal allele was not expressed in spite of the regular expression of the normal allele on the other

side, especially in the disease states of autosomal dominant transmission. In these cases, the genotype clearly showed the abnormality, but the level of the determined protein as the phenotype was mostly maintained as normal. No abnormalities were detected in known membrane proteins even in their genes in some disease states; that is, there were no abnormal protein bands or decrease in protein contents on sodium dodecylsulfate–polyacrylamide gel electrophoresis, and there were no pathognomonic mutations at the coding region or the promoter region upstream from the initiation sites of the determined membrane protein genes. Thus there is a need to elucidate the control mechanism of the expression of red cell membrane protein genes, and its contribution to the pathogenesis in disease states. Several promoters, such as GATA-1, GATA-2, and others are known to be present in the promoter region of these genes. However, in some disease states gene mutations, which are pathognomonic for the disease states, have rarely been detected [10, 11]. Thus, the possible significance of epigenetic control of membrane proteins and their related genes by epigenetic control mechanisms such as methylation, acetylation, phosphorylation, and chromatin packaging is recognized.

We have become interested in the methylation status of the promoter regions of the genes *EPB3* [12], *ELB42* [13] and *SPTB* [14] in healthy individuals, in cultured cells, and in patients with hereditary spherocytosis or hereditary elliptocytosis. *EPB3* is the gene of band 3, which is a major integral protein of red cell membranes, *ELB42* is the gene of protein 4.2, which is an anchoring protein connecting both spectrin and band 3, and *SPTB* is the gene of β -spectrin, which is a rate-limiting protein of the cytoskeletal network. The study has also been performed in the course of a genome-wide determination of methylation patterns in selected regions of the human genome. The following types of analyses were carried out: (1) direct determination of DNA methylation in all 5'-CG-3' sequences in the promoter regions by applying the bisulfite protocol of the genomic sequencing technique on DNA from mononuclear cells in peripheral blood; (2) determination of the states of methylation of the 5'-CG-3' sites in the promoter regions of the band 3, protein 4.2, and β -spectrin genes during erythroid differentiation; (3) assessment of the methylation profiles in the promoter regions of the band 3, protein 4.2, and β -spectrin genes in hereditary spherocytosis and hereditary elliptocytosis patients; and (4) determination of transcription levels of the band 3 and protein 4.2 genes in the unstimulated or stimulated UT-7 cell line, or in the erythroid cell lines K562 and AS-E2.

Patients with total deficiency of protein 4.2 carried missense mutations of the protein 4.2 gene, i.e.: (1) homozygotes with the Nippon type (424 G→A: codon 142 GCT→ACT; Thr→Ala) [15], (2) homozygotes with protein 4.2 Komatsu (523 G→T: codon 175 GAT→TAT; Asp→Tyr) [16]; or (3) compound heterozygotes with protein 4.2 Shiga (424 G→A: codon 142 GCT→ACT; Thr→Ala, and 949 C→T: codon 317 CGC→TGC; Arg→Cys) [17]. No abnormalities were detected in the sequence of the promoter regions of the protein 4.2 gene in these patients. Protein 4.2 was totally absent from red cell ghosts in these patients where the cytoskeletal network was affected, as observed by electron microscopic examinations. In addition, two patients in a hereditary spherocytosis family with frameshift mutations of the band 3 gene (band 3 Fukuyama I: codon 112–113: two nucleotide

deletion of -AG or -GA) were also studied [10]. In these cases, the contents of band 3 and protein 4.2 of the normal controls were decreased by 17 and 11 %, respectively. The phenotype of hereditary spherocytosis was transmitted with autosomal dominant inheritance.

One proband and two heterozygotes of a hereditary spherocytosis family with missense mutations of the band 3 gene (band 3 Fukuoka: 338 G→A: codon 130 GGA→AGA; Gly→Arg) have been investigated [10]. The band 3 and protein 4.2 contents of normal subjects were reduced by 30 and 60 %, respectively. The proband carried the mutation at the binding site of the band 3 protein to the protein 4.2 molecule. This mutation led to a critical reduction in the protein 4.2 content. The disorder showed autosomal recessive inheritance.

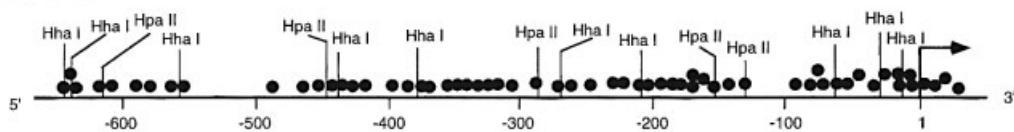
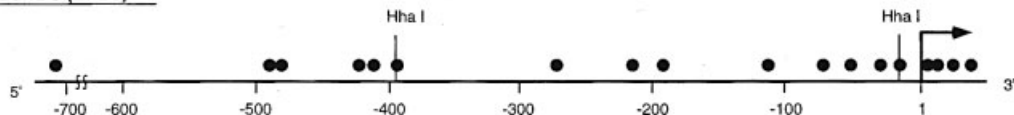
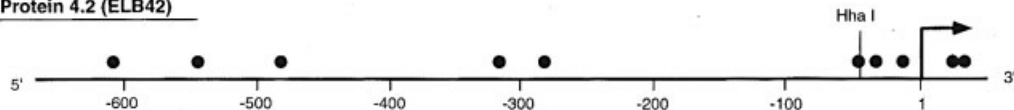
A patient with a *de novo* mutation of the β -spectrin gene (β -spectrin Nagoya) has also been studied [18]. In the patient, the β -spectrin ($\beta^{220/217}$) molecule was truncated (6205 G→T: codon 2069 GAG→TAG: Glu→stop codon), only 16.3 % of the entire β -spectrin sequence remained, although the total amount of β -spectrin was not altered. The clinical symptoms were moderate, uncompensated hemolytic anemia.

8.2

Number of 5'-CG-3' Dinucleotide Sites and Their States of Methylation

In the promoter regions of the three genes of β -spectrin, band 3, and protein 4.2, the number of 5'-CG-3' dinucleotides was most abundant (CpG-rich regions) in the β -spectrin gene, less in the band 3 gene promoter, and least in the protein 4.2 gene promoter [19] (Fig. 8.1). The results indicate that the 5'-CG-3' dinucleotides were most preserved in the β -spectrin gene. The 5'-CG-3' dinucleotides in the promoter region of the β -spectrin gene were completely unmethylated (Fig. 8.2). These observations demonstrate that 5'-CG-3' dinucleotides were most preserved in *SPTB*. The genetic functionality of β -spectrin as one of the most essential membrane proteins in the construction of the cytoskeletal network should remain uncompromised. Thus, it is not surprising to find all 5'-CG-3' dinucleotides in this promoter in the unmethylated state. In contrast, the promoters of the band 3 gene and protein 4.2 gene were generally hypermethylated.

When the bisulfite protocol of the genomic sequencing method was applied, there was heterogeneity to the extent that there was methylation of each 5'-CG-3' position in this DNA segment [19]. In the DNAs of peripheral mononuclear cells, the 5'-CG-3' sites in the promoter of the band 3 gene were essentially heavily methylated although considerable variability from site to site existed (Fig. 8.2). Some sites of 5'-CG-3' dinucleotide sequences consistently showed levels of methylation lower than the other sites. The two distinctly hypomethylated sites were observed in the band 3 promoter. While the significance of this observation is as yet unknown, it underlines the notion of highly specific and unique patterns of DNA methylation in the human genome and its possible contribution to the architecture of chromatin. In the regions of the promoter and exon 1 of the protein 4.2 gene, 38–100 % of the 5'-CG-3' sequences were methylated [19] (Fig. 8.2). It is also ap-

***β*-Spectrin (SPTB)****Band 3 (EPB3)****Protein 4.2 (ELB42)**

Nucleotide Number

Initiation
Site

Figure 8.1 Number and location of 5'-CpG-3' sites and endonuclease cleavage sites in the promoter regions of the genes of red cell membrane proteins (*β*-spectrin, band 3, and protein 4.2).

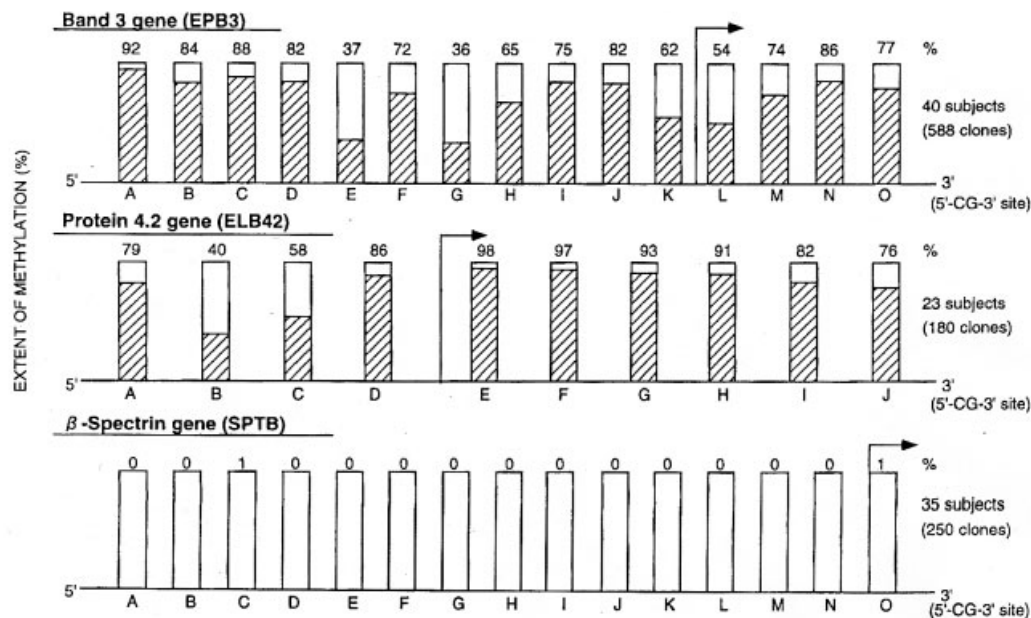


Figure 8.2 The extent of methylation (%) at the 5'-CpG-3' sites of the promoter region of genomic DNAs of band 3, protein 4.2 and *β*-spectrin obtained from normal human mononuclear cells in peripheral blood.

parent that mononuclear cells from peripheral blood and erythroid cells, which were enriched to 92 % on day 15 of the second phase in the two-phase liquid culture method, did not significantly differ in the extent of the 5'-CG-3' methylation in the genome segments analyzed. Hence, the results of analyses on peripheral mononuclear cells were also representative for cells of the erythroid lineage.

8.3

Transcriptional Activity of *EPB3* and *ELB42* in Various Human Cell Types

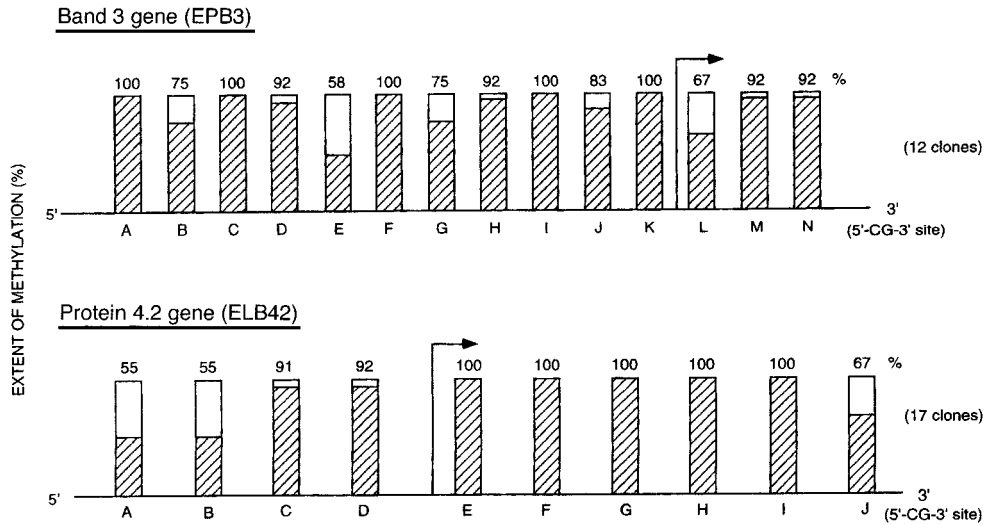
Reverse transcription and PCR amplification of transcription products are the most sensitive methods for detecting transcripts in the total RNA of human cells, although the method has limits in precisely quantitating the relative amounts of transcripts. Total RNA was isolated from erythroid committed cells of K562 and AS-E2 (provided courtesy of Professor M. Tomonaga, Nagasaki University, Japan) [20] in culture, from the erythroblastic cell line UT-7 (provided courtesy of Professor N. Komatsu, Jichi Medical School, Japan) [21, 22] treated with EPO (UT-7/EPO), or from the latter cell line after stimulation with granulocyte macrophage colony stimulating factor (GM-CSF). Upon erythropoietin treatment of UT-7 cells (UT-7/EPO), the band 3 gene and protein 4.2 gene were transcribed, this being apparent from the strong PCR signals and the verified authentic nucleotide sequences of both PCR products. After GM-CSF stimulation of UT-7 cells (UT-7/GM), band 3 transcripts were not found. The protein 4.2 gene transcript was barely identifiable. Both genes were transcribed in the UT-7/GM cell lines, when GM-CSF was replaced by EPO (UT-7/GM/EPO). In the erythroid cell lines K562 and AS-E2, transcription of the band 3 gene, but not of the protein 4.2 gene, was detected. It was concluded that, in UT-7/EPO or UT-7/GM/EPO cells, the band 3 gene and protein 4.2 gene were transcribed [19]. GM-CSF treatment by itself eliminated transcription almost completely. In cell lines K562 and AS-E2, the band 3 gene, but not the protein 4.2 gene, was transcribed [19].

8.4

Methylation in *EPB3*, *ELB42*, and *SPTB* Promoters During Erythroid Development and Differentiation

As described previously, the spectrins are expressed in early erythroid precursors, such as in pronormoblasts [23, 24]. Band 3 was expressed nearly at the same stage, protein 4.1 follows, and protein 4.2 was expressed at the very late erythroblast stage, when studied by the two phase liquid culture method, or by utilizing erythroblasts from normal human bone marrow (Fig. 7.9). Hence, the protein 4.2 gene appeared to be a suitable model to study the role of 5'-CG-3' methylation in its promoter for the sequential expression of the gene during human erythroid development and differentiation [23, 24]. In mononuclear cells from peripheral blood, these dinucleotides in the protein 4.2 promoter were methylated (Fig. 8.2). The cells on day 0 of the

(A) Extent of DNA methylation (%) at the 5'-CG-3' sites of the promoter Region of the band 3 and protein 4.2 genes in erythroid cells at day 15 of the second phase by the two-phase liquid culture method.



(B) Extent of DNA methylation (%) in the promoter region of the band 3 and protein 4.2 genes in cell lines (UT-7/EPO) derived from the cell line UT-7 after the addition of erythropoietin.

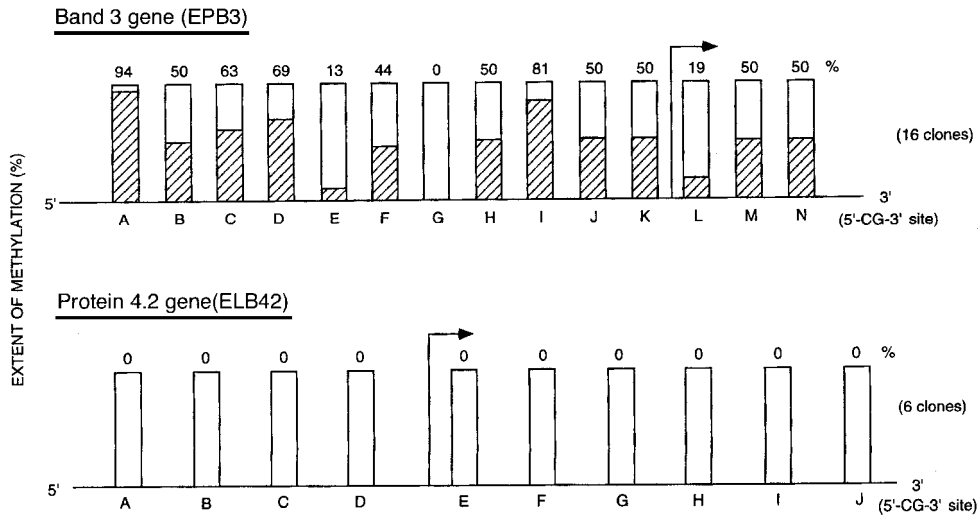


Figure 8.3 State of methylation. (A) Extent of DNA methylation (%) in the 5'-CG-3' sites of the promoter region of the band 3 and protein 4.2 genes in erythroid cells at day 15 of the second phase by the two phase liquid culture method. (B) Extent of DNA methylation (%) in the promoter region of the band 3 and protein 4.2 genes in cell lines (UT-7/EPO) derived from the cell line UT-7 after the addition of erythropoietin.

second phase (1 % erythroblasts) as well as on day 15 with 92 % erythroblasts of the two-phase liquid culture method demonstrated that the 5'-CG-3' dinucleotides in the protein 4.2 gene were methylated (Fig. 8.3A) to the same extent as those in white cells from peripheral blood [19].

A UT-7 cell line (UT-7/EPO) was erythroid-committed in the presence of erythropoietin (EPO), contrary to the non-erythroid control cell line UT-7/GM with erythropoietin and GM-CSF. In the erythroid committed cell line UT-7/EPO, the 5'-CG-3' dinucleotides in the band 3 gene promoter were methylated about 50 % [19]. In contrast, in the protein 4.2 gene promoter, the 5'-CG-3' dinucleotides were completely unmethylated at the early erythroid precursor stage, that is, at the stage preceding the proerythroblasts [19] (Fig. 8.3B). In a differentiation-dependent fashion, the 5'-CG-3' dinucleotides could turn from unmethylated to methylated at the stage before proerythroblasts. In the β -spectrin gene promoter, the 5'-CG-3' dinucleotides remained consistently unmethylated throughout the entire cycle of erythroid differentiation [19].

Thus, the 5'-CG-3' dinucleotides of the protein 4.2 gene promoter were totally unmethylated in the early erythroid precursors of cell line UT-7/EPO, even in the presence of erythropoietin [19] (Fig. 8.3). During erythroid development and differentiation, mRNA from the protein 4.2 gene was not expressed [24]. This mRNA arose only on day 3 of the second phase in the two-phase liquid culture method [24]. At this stage of differentiation, the 5'-CG-3' dinucleotides in the protein 4.2 gene promoter were methylated [19]. Membrane protein 4.2 molecules were expressed at the very late erythroblast stage [24]. In the β -spectrin gene pro-

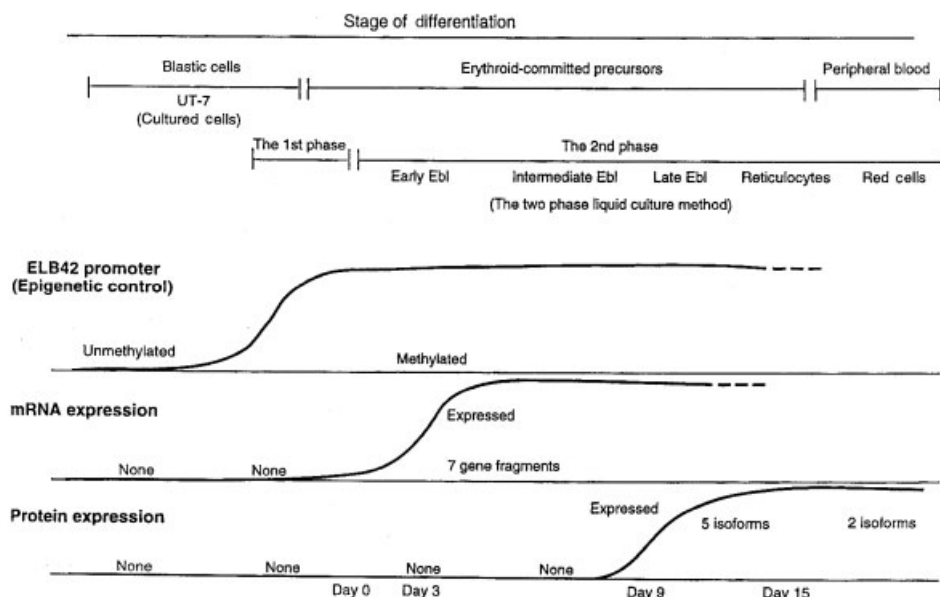


Figure 8.4 The sequential events of the expression of protein 4.2 during differentiation of human erythroid cells.

moter and band 3 gene promoter, we could not evaluate the contribution of methylation to the sequential expression of mRNA and their membrane proteins, because these membrane proteins were already expressed in early erythroid precursors.

Summarizing the state of promoter methylation, gene transcription, and protein expression of the band 3 and protein 4.2 genes at different stages of differentiation and maturation for cells of the erythroid lineage (Fig. 8.4), the activity of the methylated promoter segments of the genes *EPB3* and *ELB42* might be explained by trans-acting transcription-stimulating factors present in UT-7 cells upon erythropoietin stimulation. In a very different system of mammalian cells and adenoviral promoters, it has previously been demonstrated that the 13S-transcribed genes of the early E1A region of human adenovirus type 2 (Ad2) are capable of overcoming (at least in part) the inactive state of the 5'-CCGG-3' methylated E2A promoter of the Ad2 genome, which has been studied in detail for the effect of sequence-specific promoter methylation on promoter activity [3–8].

8.5

Methylation in the Disease States

There are numerous reports on mutations in red cell membrane disorders. Most of these mutations affect the coding regions of the affected genes. The most frequent red cell membrane disorder is hereditary spherocytosis. These mutated alleles are frequently not expressed [10, 25, 26]. A mechanism by which a mutated allele is silenced would be beneficial for the organism, because the mutated allele may have deleterious effects [27–32]. One of the candidates for the repression of a mutated gene is methylation. The following patients were studied. (1) Three types of total deficiency of protein 4.2 (protein 4.2 Nippon, protein 4.2 Komatsu, and protein 4.2 Shiga). The probands chosen for analyses were homozygotes or compound heterozygotes *in trans* of the mutated *ELB42* with its missense mutations. (2) A proband with band 3 Fukuyama, where the patient was heterozygous for the frameshift mutation of *EPB3*. (3) A homozygous patient with a missense mutation of *EPB3* (band 3 Fukuoka). (4) A heterozygous patient with a β -spectrin anomaly (β -spectrin Nagoya) with the frameshift mutation of *SPTB*. (5) Five patients with hereditary spherocytosis of unknown etiology, in whom no gene mutations were present regarding *SPTB*, *EPB3*, *ELB42*, and the ankyrin gene.

In homozygotes with three different types of total protein 4.2 deficiency, the states of methylation in the 5'-CG-3' dinucleotides of the *EPB3* and *ELB42* were the same as those in normal individuals [19]. In the heterozygous proband of β -spectrin Nagoya, there was no methylation observed, as described in normal subjects [19]. Despite the presence of the mutated allele, which was expressed with the aberrant β -spectrin molecule (approximately 16 % of total β -spectrin content), the promoter remained unmethylated [19]. In a heterozygous proband with band 3 Fukuyama I, a frameshift mutation in *EPB3*, the 5'-CG-3' dinucleotides in *EPB3* and *ELB42* promoters remained methylated, as in normal subjects [19]. Lastly, no significant changes were observed in the states of methylation of *EPB3*, *ELB42*, and *SPTB* promoters in the patients with hereditary spherocytosis without known gene mutations [19].

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Disease States of Red Cell Membranes: Their Genotypes and Phenotypes

A clue to the pathobiology and diagnosis of red cell membrane disorders has been provided mainly through red cell shape abnormalities, although the molecular pathology and the characterization of mutations of membrane proteins in relation to the underlying changes of these disorders have been studied extensively because of the progress made in the characterization of the structure and function of red cell membrane proteins and their genes (Tables 1.1 and 1.2). In clinical hematology, the red cell shape abnormalities are still widely utilized for first step-diagnoses before further detailed characterization of the phenotype and genotype of these disorders. Therefore, the following Chapters (10 to 13) categorize the disease states of red cell membranes according to morphological and clinical phenotypes: (i) hereditary spherocytosis (HS); (ii) hereditary elliptocytosis (HE) including hereditary pyropoikilocytosis (HPP), and Southeast Asian ovalocytosis (SAO); (iii) hereditary stomatocytosis (HSt); (iv) acanthocytosis, and (v) spur cells and target cells.

The molecular abnormalities specific to each membrane protein and lipid are also outlined in the subsequent Chapters (Chapters 14–17).

The structure and functions of normal red cell membranes that have already been described in detail in the previous Chapters 2–8, should assist in the understanding of the descriptions of the pathological states of red cell membranes in the following Chapters (10–17).

An interesting and useful hypothesis has been proposed that red cell membrane disorders can be categorized into two groups, that is, (1) abnormalities in vertical interactions of membrane components, and (2) those in their horizontal interactions (Fig. 2.2) [1].

The vertical interactions consist mainly of spectrin–ankyrin–band 3 interactions, the protein 4.1–glycophorin C linkage, and the interactions of the skeletal proteins, that is the membrane lipids (especially, PE and PS) with a negative charge, which are located at the inner leaflet of the lipid bilayer. The role of these interactions has been speculated as being to stabilize the lipid bilayer of the red cell membranes. HS is considered as the most typical example in the disorder of the vertical interactions. In fact, one common feature of red cell abnormalities in HS is a weakening of the vertical contacts between the skeletal network

and integral proteins in the overlying lipid bilayer. As a result, the lipid bilayer becomes destabilized and loses membrane lipids from the bilayer as skeleton-free lipid vesicles. Through this process, the membrane surface area is reduced to produce microspherocytosis. This hypothesis is indeed attractive. However, the interactions between membrane proteins are not as simple as this, but are more complex than can be accounted for by this model of vertical interaction alone.

The horizontal interactions involve the self-association of spectrin to form its tetramers, which are the most important component of the cytoskeletal network, and the interactions of the distal ends of the spectrin tetramers with protein 4.1 and actin, which result in the formation of the junctional complex. These horizontal interactions are proposed as a way of supporting the structural integrity of red cells after their exposure to shear stress. HE is considered as the most typical example in the disorder of the horizontal interactions. The weakened horizontal interactions produce a disruption of the skeletal lattice, due to skeletal instability especially under the shear stresses, and make the red cell shapes more ovalocytic and elliptocytic.

Although this hypothesis is basically relevant to the understanding of the pathogenesis of membrane abnormalities, it is however not applicable to all red cell membrane disorders. For example, a critical role of protein 4.2 was not considered at the time of this proposal, because the exact functions of this protein were not elucidated at that time. Total deficiency of protein 4.2 is now known to demonstrate serious abnormalities both of the vertical interactions (such as the disorganization of band 3) and also of the horizontal interactions (such as the instability of the cytoskeletal network) at the same time. In addition, in HE, the ovalocytic red cells of SAO with the mutated band 3 protein are rigid and hyperstable rather than unstable, as seen in a conventional HE. β -Spectrin abnormalities are observed in HE, but also in some patients with HS (Fig. 1.5). There are many other known exceptions to the strict application of this hypothesis for all the red cell membrane disorders in general. The details are discussed in the following sections.

9.1

Incidence of Red Cell Membrane Disorders

A detailed report on the incidence of red cell membrane disorders has never been published, because no well-controlled field studies have been performed. However, a nationwide survey on the incidence of hemolytic anemias was carried out in 1974 under the direction of the Project for Hemolytic Anemias of the Japanese Ministry of Health and Welfare [2]. Two hundred and fifty-six cases of congenital hemolytic anemias were registered by university hospitals and major hospitals in Japan. Although the absolute number of such patients registered was fairly limited, the relative incidence of hemolytic anemias of different pathogeneses could be demonstrated. As a result of this study survey, most of the congenital hemolytic anemias in Japan were recognized as membrane disorders, predominantly hereditary spherocytosis (181 cases; 70.7% of the total). The incidence of HE (seven cases; 2.7%)

appeared fairly low, because the survey was limited only to the hemolytic anemias with overt symptoms. Other membrane disorders, such as hereditary stomatocytosis and others, were not itemized in this particular survey. The incidence of hemoglobinopathies (12 cases; 4.7%) and of enzymopathies (15 cases; 5.9%) was extremely low, in contrast to the much higher incidence of red cell membrane disorders (73.4 % of the total congenital hemolytic anemias).

During the last two decades, the pathogenesis and molecular mechanisms of red cell membrane disorders have been elucidated extensively, and the classification of these disorders *per se* appears to require a reevaluation based on biochemical, medical genetic, and molecular electron microscopic findings.

For the last 26 years, the red cell membrane disorders of 1014 patients from 605 families in the Japanese population referred to us randomly for detailed studies have been examined in a single laboratory in the Division of Hematology of Kawasaki Medical School using the same consistent standardized protocol [3]. Membrane protein abnormalities were identified in 825 patients (81.4 % of the total) from 438 families and membrane lipid abnormalities were found in 41 patients (4.0 %) from 27 families. Disorders of unknown etiology were diagnosed in 148 patients (14.6 %) from 140 families (Table 9.1). Therefore, HS appears to be the most common red cell membrane disorder in the Japanese population [3].

Most of the protein 4.2 (P4.2) anomalies (34 patients from 20 families) were incorporated into the category of HS, except for seven cases of two traits with doublet P4.2 anomalies, which demonstrated hereditary stomatocytosis (HSt) [3].

Table 9.1 Incidence of hereditary red cell membrane disorders in Japanese population, as studied at Kawasaki Medical School (1975–2000).

Disorder	No. of families	No. of cases	Percent (%)
Membrane protein abnormalities	438	825	81.4
Hereditary spherocytosis (HS)	303	581	57.3
Hereditary elliptocytosis (HE)	68	137	13.5
Hereditary stomatocytosis (HSt)	64	104	10.2
Glycophorin abnormalities ^a	3	3	0.3
Membrane lipid abnormalities	27	41	4.0
Hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA)	19	31	3.1
Congenital LCAT deficiency	1	1	0.1
Congenital β -lipoprotein deficiency (Acanthocytosis)	6	8	0.8
Congenital α -lipoprotein deficiency (Tangier disease)	1	1	0.1
Unknown etiology	140	148	14.6
Total	605	1014	100

^a Glycophorin (GP) abnormalities include each one case of GP-A deficiency (En[a–]), GP-B deficiency, and Miltenberger V anomaly. LCAT, lecithin: cholesterol acyltransferase. Total deficiency of protein 4.2 (34 patients from 20 families) was incorporated into the category of HS, except for seven cases of two traits with doublet protein 4.2 anomalies, which demonstrated HSt.

Before discussing the incidence of red cell membrane disorders in detail, it should be noted that several biases exist in this study. First of all, the results are totally based on the patients at our own clinic or those referred to us from other institutions all over Japan for detailed investigation, and not on a field survey study. Secondly, blood specimens of suspected red cell membrane disorders were sent to us for two major purposes, that is: (1) to obtain an adequate diagnosis for these cases, and (2) to obtain more information on the pathogenesis of specific patients (by precise protein chemistry and gene analysis), even when a diagnosis had already been made at the initial institutions. Thirdly, the diagnosis on red cell membrane disorders is dependent on the criteria used to make that diagnosis. Fourthly, the determined incidence of red cell membrane disorders is totally dependent on the screening protocol. In our laboratory, membrane lipid analysis and membrane monovalent cation transport have been incorporated into the protocol since 1975, when we initiated membrane studies. Therefore, red cell membrane disorders with membrane lipid abnormalities or membrane transport abnormalities have been intensively examined in our laboratory.

Many biases are obviously present as mentioned above, and there should be severe reservations because of these factors. However, our outline of the relative incidence of red cell membrane disorders should still be useful and informative for clinical hematology.

The cases studied were essentially classified on the basis of the findings from clinical hematology, especially through red cell morphology in the peripheral blood. When some specific findings were obtained by detailed investigation, the diagnosis tended to be made based on those specific observations, namely for specific membrane protein abnormalities (spectrin, band 3, protein 4.1, protein 4.2, etc.), and membrane lipid abnormalities, such as hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCA), congenital lecithin: cholesterol acyltransferase (LCAT) deficiency, and others.

The last factor to be considered is the progress in red cell membrane research. Even in the early stage of our investigation in 1975, our routine screening system already included red cell morphology (mainly by scanning electron microscopy: SEM), SDS-PAGE for membrane proteins, membrane lipids, and sodium transport (influx and efflux, red cell sodium and potassium contents, and the enzymatic activities of ATPases). Spectrin kinetics were incorporated into our study system around 1984. Biophysical studies (ektacytometry, electron spin resonance, etc.) were added to the protocol in 1982, and band 3 kinetics were begun in 1990. Gene analysis also began in 1990. Therefore, better elucidation of the molecular abnormalities in these red cell membrane disorders has gradually become possible at different levels of investigation.

The relative incidence of red cell membrane disorders studied in our laboratory was basically similar to that in other laboratories in other countries (the United States, France, etc.) (Professors Jean Delaunay, Lyon, currently in Paris, France and Jiri Palek, Boston, USA: personal communications), except for the higher incidence of membrane lipid abnormalities (especially HPCA), and hereditary stomatocytosis, which may be due to the fact that membrane lipids and sodium fluxes

are not included in the routine protocol in these foreign laboratories [3]. Primary protein 4.2 anomalies are also specific to Japan [4, 5]. Only a few families have been discovered in a non-Japanese population, and these were found in Tunisia, Portugal, Italy and France.

Our large scale studies on the phenotypic expression of hereditary stomatocytosis were unique. It was surprising that in 27 % of the patients with this disorder, there was normal sodium influx, contrary to the commonly-held belief that stomatocytosis could be related to cell water abnormalities due to impaired cation transport [6]. Stomatocytosis with mildly increased sodium influx was difficult to differentiate from classical HS. The patients without microspherocytosis were classified as hereditary stomatocytosis in addition to other findings in clinical hematology. Therefore, in general, stomatocytosis does not necessarily imply impaired membrane transport, to which band 7 appeared not to be directly related.

As for the molecular abnormalities of the red cell membrane disorders of the patients studied, many anomalies were identified at the level of protein chemistry.

The most striking difference was present in the abnormalities of α -spectrin (Fig. 1.5). In Japan, only one family (HE $\alpha^{1/74}$) was detected in the HE studied [3], contrary to the findings in other countries, where numerous abnormalities of α -spectrin have been found, and where around two-thirds of HE could be due to α -spectrin mutations.

The frequency of α^{LELY} polymorphism in the normal Japanese population was nearly identical to that in France [7]. Since the low expression gene (α^{LELY}) was present in Japan to the same extent as that in France, one could expect the same increased expression of α -spectrin mutation in Japan. Fortunately, however, the frequency of α -spectrin gene anomalies appeared extremely low in Japan. By contrast, the prevalence of β -spectrin anomalies in Japan appeared to be the same as that in other countries, because three β -spectrin mutations, spectrin Tokyo ($\beta^{220/216}$), spectrin Le Puy in Yamagata ($\beta^{220/214}$) and spectrin Nagoya ($\beta^{220/217}$), were detected in Japan [3] amongst the 20 β -spectrin mutations reported worldwide.

It is also interesting that most of the cases of HE (81 %) in Japan appear to be due to a partial protein 4.1 deficiency [3], compared with those in other countries, where only one-third of the HE cases have been protein 4.1-deficient.

There is no question that protein 4.2 anomalies are definitely unique to Japan [4, 5]. Several phenotypic differences exist among the families with protein 4.2 deficiency. The protein 4.2 deficiency of the Nippon type (GCT→ACT at codon 142), which is a major category of protein 4.2 deficiency in Japan, demonstrates ovalosomatocytosis, contrary to the other types of protein 4.2 deficiency, in which their phenotypes appeared as HS [8]. Generally speaking, homozygotes of a point mutation (GCT→ACT at codon 142 in protein 4.2 gene) demonstrate complete deficiency of protein 4.2 in red cells. Heterozygotes, on the other hand, show no deficiency of protein 4.2 and are clinically normal.

9.2 Screening Procedures for Membrane Disorders

The patients from the out-patient clinic of the Division of Hematology, Department of Medicine, Kawasaki Medical School and those referred to us from institutions all over Japan were studied basically by the determined protocol (Table 9.2). They were initially screened for congenital disorders with red cell membrane abnormalities from the standpoints of clinical history, clinical hematology, particularly with re-

Table 9.2 Screening protocol for red cell membrane disorders at Kawasaki Medical School.

(1) <i>Red cell morphology</i>	Phase contrast microscopy Scanning electron microscopy Intramembrane particles (IMP) (freeze-fracture method) Cytoskeleton (quick-freeze deep-etching method, negative staining, and surface replica method) Field-emission scanning electron microscopy (FE-SEM) Immuno-electron microscopy
(2) <i>Red cell membrane proteins</i>	SDS–PAGE (Fairbanks, Laemmli) Extracted spectrins Dimer–tetramer conversion Tryptic digestion Two-dimensional peptide mapping Inside-out-vesicles (IOV) Binding studies with ankyrin, protein 4.2 Cleavage studies Oligomerization of band 3 (HPLC)
(3) <i>Biophysical studies</i>	Membrane deformability (ektacytometry) Mechanical stability Fluorescence recovery after photobleaching (FRAP) for band 3
(4) <i>Membrane lipids</i>	Thin layer chromatography (TLC) Fatty acid composition Membrane fluidity (electron spin resonance, ESR)
(5) <i>Membrane transport</i>	Na ⁺ flux (influx, efflux) Red cell Na ⁺ , K ⁺ content Enzymatic activities of ATPases Anion transport (SO ₄ ²⁻ , PO ₄ ³⁻)
(6) <i>Gene analysis</i>	mRNA content Universal cDNA by reverse transcriptase–PCR Genomic DNA Epigenetic control mechanism by DNA methylation
(7) <i>Development and expression of membrane proteins</i>	Two-phase liquid culture system using BFU-E in peripheral blood Expression of mRNA and membrane proteins Intracellular expression of membrane proteins
(8) <i>Surface markers</i>	Blood group antigens Sialic acid content

gard to abnormal red cell morphology, and other specific laboratory examinations. Most of these patients demonstrated increased hemolysis except for some patients with hereditary elliptocytosis and hereditary stomatocytosis. Hemoglobinopathies and enzymopathies, if suspected, were excluded from this study by direct biochemical measurements, although previous studies have shown these disorders to be rare in Japan [2].

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10

Hereditary Spherocytosis (HS)

10.1

Definition and History

Hereditary spherocytosis (HS) is a clear indicator of congenital red cell membrane disorders in the Caucasian and Japanese populations. This disorder is characterized by the presence of microspherocytosis on a smear from peripheral blood (Fig. 10.1). The most common form is a hemolytic anemia of autosomal dominant inheritance with variable clinical severity.

In 1871, Vanlair and Masius in Liège of Belgium [1] encountered a family with jaundice and splenomegaly, in which small and spheroid red cells (4 μm in diameter) on their peripheral smears were observed under a light microscope (Fig. 1.2). They reported their observations at the Belgium Royal Academy of Medicine as “de la microcythémie”. This report is believed to be the first description of HS to speculate that the pathogenesis of the jaundice lay in the increased destruction of the “globules atrophiques” as discussed in Section 1.2.

In 1893, Wilson and Stanley in England reported a hereditary disorder with anemia, splenomegaly, jaundice, and gallstone episodes, although no morphological characteristics of the red cells were described.

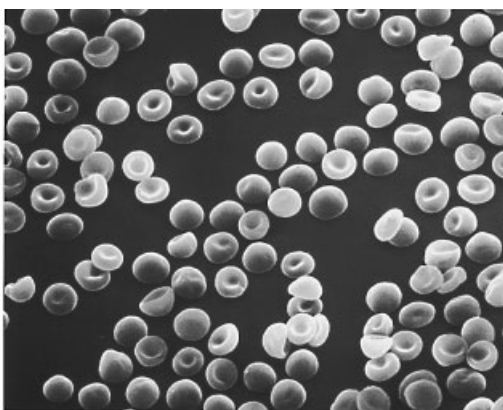


Figure 10.1 A scanning electron micrograph of typical microspherocytosis in peripheral blood of a patient with hereditary spherocytosis.

Oskar Minkowski, who was a German physician of Russian descent, in 1900 reported on eight members of a family of “hereditärer chronischer Ikterus” with life-long jaundice and marked splenomegaly, in spite of the fact that he described normal red cell morphology in these family members.

The most impressive observation was made by Anatole Chauffard (France) in 1907 [2]. He saw a 24 year old man with congenital jaundice, who showed gallstone episodes, marked anemia, and positive urobilinuria. He first examined the so-called osmotic fragility test utilizing the patient’s red cells, this test having initially been invented by Vaquez. Three affected members of this family with “l’ictère congenital de l’adulte” demonstrated a marked reduction in osmotic resistance in their red cells. Furthermore, he described that the mean red cell diameter in their red cells was much smaller ($5.89\text{ }\mu\text{m}$: $7.5\text{--}4.0\text{ }\mu\text{m}$) than in normal subjects, indicating the presence of microcytosis, which was osmotically less resistant. Thus, his description is considered to be the first observation on the abnormal membrane function in HS red cells.

Gänsslen completed clinical observations on HS in 1922 [3]. He described carefully and thoroughly the “hämolytischer Ikterus”, with respect to autosomal dominant inheritance, factors accelerating hemolysis (common cold, infections, menstruation, pregnancy, etc.), and the presence of increased erythropoiesis. He classified this disorder into the three clinical forms, that is: (1) klassische (polysymptomatische) Form, (2) oligo-oder-monosymptomatische Form, and (3) kompensierte Form (ohne Ikterus und Anämie). In addition, he pointed out the presence of sporadic cases, which were speculated to be due to a *de novo* mutation, and also a successful splenectomy as the treatment of this disorder. Therefore, an overview of HS had already been established in the 1920s from the standpoint of both red cell membrane abnormalities and a splenic contribution to the microspherocyte formation.

10.2

Clinical and Laboratory Findings

A typical clinical picture of HS [4–6] is composed of two factors, that is, (1) increased hemolysis, and (2) enhanced erythropoiesis as a compensatory mechanism for increased hemolysis. Increased hemolysis indicates the presence of jaundice and gallstones due to hyperbilirubinemia especially for the indirect form, and splenomegaly as the site of increased red cell sequestration and destruction. Enhanced erythropoiesis demonstrates increased reticulocytosis and the presence of nucleated red cells in the peripheral blood smear. However, the most critical indicator is the presence of microspherocytosis in the peripheral blood smear (Fig. 10.1). In this disorder, the presence or absence of anemia or the extent of anemia, if present, is dependent on the balance between the extent of increased hemolysis and that of enhanced erythropoiesis.

A nationwide survey of HS was carried out by the Committee for Studies on Hemolytic Anemias for the Japanese Ministry of Health and Welfare in 1974–1977 [7].

At the time of diagnosis, jaundice was observed in 86 % of the 144 HS patients, splenomegaly in 76.4 %, and gallstone in 22.2 %, respectively. It is interesting to note that no cases of leg ulcers or the appearance of extramedullary hematopoietic tumors were observed, contrary to its higher incidence in sickle cell anemia and patients with severe thalassemias. The age of the first visit to medical clinics was 17.0 ± 15.2 years. The size of the spleen was 2.3 ± 1.5 finger-widths below the right costal margin [7].

In the peripheral blood, red cell counts were $3.07 \pm 0.62 \times 10^6 \mu\text{L}^{-1}$, hemoglobin (Hb) $9.9 \pm 2.1 \text{ g dL}^{-1}$, hematocrit (Hct) $27.8 \pm 5.4 \%$, reticulocytes $12.2 \pm 10.0 \%$ (or $0.37 \pm 0.32 \times 10^6 \mu\text{L}^{-1}$), respectively. The mean corpuscular volume (MCV) of red cells and the mean corpuscular hemoglobin concentration (MCHC) was $90 \pm 12 \text{ fL}$ and $36.0 \pm 3.8 \%$, respectively, in the HS patients with marked spherocytosis, and $96 \pm 12 \text{ fL}$, and $35.0 \pm 3.2 \%$, respectively, in those with minimal spherocytosis [7].

Considering the extent of increased hemolysis, total bilirubin and indirect bilirubin in the plasma of the HS patients, these were $4.00 \pm 2.12 \text{ mg dL}^{-1}$ and $3.16 \pm 2.09 \text{ mg dL}^{-1}$, respectively [7].

As for the extent of erythropoiesis of the bone marrow, the nucleated cell counts (NCC) was $0.34 \pm 0.15 \times 10^6 \mu\text{L}^{-1}$. The erythroid cells were $51 \pm 16 \%$ (or $0.18 \pm 0.10 \times 10^6 \mu\text{L}^{-1}$) of these nucleated marrow cells. The apparent half-life ($T_{1/2}$) of red cell survival by the Cr^{51} method was significantly decreased (9.9 ± 4.0 days) in the HS patients [7].

Based on the extent of the severity of clinical manifestations, HS can be categorized into three forms, that is, a typical classical form, a mild form including a carrier state, and a severe form [4–6].

Regarding a typical classical form of HS, the patients are relatively asymptomatic, although mild jaundice owing to the presence of increased hemolysis is usually present. In most patients, splenomegaly can be detected, as mentioned before. Gallstones are also observed in some of the HS patients. The extent of anemia is variable from mild to moderate, and may be absent when the increased hemolysis is compensated for by the enhanced erythropoiesis.

In some patients with the mild form of HS, the reticulocyte count is normal with a normal bilirubin level. Microspherocytosis is minimal or barely observed. The osmotic fragility test can only detect the presence of this disorder, or the silent affected members may only be identified by thorough examination of other affected family members.

In severe cases of HS, red cell transfusion may be required. There are only a few patients with HS of the severe form, mainly in Japan [7].

As regards laboratory evaluation, the extent of the anemia is not diagnostic, because anemia may not be present when the increased hemolysis is fully compensated for by the enhanced erythropoiesis. The mean corpuscular volume (MCV) of HS red cells could be expected to be low, because of the presence of microspherocytosis. However, as described before, MCV can be only slightly low or even normal, because the increased number of reticulocytes, which are usually larger in size than normal red cells, elevates the MCV value. In contrast, the elevated mean corpuscular hemoglobin concentration (MCHC), which reflects a loss of

surface area to cell volume ratio with cellular dehydration, is an excellent indicator to detect the presence of microspherocytosis. Increased enzymatic activity of lactic dehydrogenase (LDH) is not diagnostic in a mild form of HS, because the extent of the increment is usually minimal or the LDH activity may be normal.

The identification of the presence of microspherocytosis is definitely diagnostic (Fig. 10.1). It is not difficult to identify the characteristic cell shape on the peripheral blood film. The microspherocytes are small and spherical in shape, lack a central pallor, and appear more intensely hemoglobinized. Although microspherocytosis is an indicator of HS, various abnormal red cells on the stomatocytic transformation (Fig. 2.4), that is, from discocytes, through discostomatocytes and stomatocytes, and finally to stomatospherocytes and spherocytes, are observed on the peripheral blood smear.

On some occasions, one may have difficulty in identifying microspherocytes on the dry smear obtained from the peripheral blood. Observation of the HS red cells utilizing the “wet film” of the blood specimens is an excellent solution to this problem. A drop of red cell suspension, which is diluted with its autologous plasma, is placed on the glass slide and the red cell shape examined by phase-contrast microscopy. One may be able to identify microspherocytes in the three-dimensional image, and also to differentiate various cell shapes clearly. This method is also critically important to differentiating HS from hereditary stomatocytosis (see Chapter 12), in which the microspherocytes are basically absent.

Additional morphological features have been reported in some HS patients, for examples, pincerred (mushroom-shaped) red cells in HS with band 3 deficiency [8, 9], acanthocytic spherocytes [10] in HS with β -spectrin anomalies, and ovalo-stomatocytosis in protein 4.2 deficiency, especially of a Nippon type [11].

Amongst the various laboratory procedures for diagnosing HS, the osmotic fragility test is the most critical one. The basic concept of this test was first established by the observation made by Chauffard as early as 1907 [2]. This test measures the *in vitro* lysis of red cells suspended in solutions of decreasing osmolarity. Since the normal red cell membrane is freely permeable to water, the red cell increases its volume in hypotonic solutions progressively, until a critical hemolytic volume is attained, and it then ruptures. The amount of hemoglobin, which escapes into the supernatant solution through a hole, is determined spectrophotometrically to estimate the osmotic fragility of the red cell membrane. When a loss of membrane and the ensuing surface deficiency are present, the critical hemolytic volume of spherocytes is considerably lower than that of normal red cells. As a result, these spherocytic cells hemolyze more than normal red cells when they are suspended in hypotonic sodium chloride solutions. It should be noted that any pathological conditions demonstrating spherocytosis could show an increased osmotic fragility. Therefore, an increased osmotic fragility does not guarantee the diagnosis of HS, such as hereditary stomatocytosis of a hydrocytosis type (see Section 12.2), in which water permeability is enhanced tremendously in these red cells.

The routine osmotic fragility test is composed of two sections. The first one is a test utilizing a fresh red cell suspension, and the second is with red cells that have been incubated aseptically at 37 °C for 24 h. Defibrinated blood should be used.

Characteristically, osmotic fragility curves from unsplenectomized patients with HS show a “tail” of very fragile cells. There are two populations of cells, the very fragile and the normal or slightly fragile. After splenectomy, the red cells are more homogeneous from fragile to normal. The osmotic fragility of red cells after sterile incubation at 37 °C for 24 h is also a reflection of their surface area to cell volume ratio, but the factors which alter this ratio are more complicated than in fresh red cells. The increased osmotic fragility of normal red cells is mainly caused by swelling of the cells associated with an accumulation of sodium which exceeds the loss of potassium. The cation exchange is determined by the membrane properties of the red cell that control the passive flux of ions and the metabolic competence of the cell, which determines the active pumping of cations against concentration gradients. During incubation for 24 h the metabolism of the red cell becomes stressed and the pumping mechanisms tend to fail, one factor being a relative lack of glucose in the medium. Therefore, the osmotic fragility of abnormal HS red cells increases the abnormality after incubation. The minimal abnormality of osmotic fragility in HS of a mild form manifests itself after the incubation of the red cells at 37 °C for 24 h, even if these red cells do not demonstrate any abnormality in the fresh condition without any incubation.

Osmotic gradient ektacytometry is also useful (see Section 2.3.4.2), although this is only available in specialized laboratories.

The autohemolysis test is based on the increased spontaneous hemolysis of red cells incubated under sterile conditions without glucose at 37 °C for 48 h. This test has been widely utilized in clinical hematology to differentiate HS from red cell glycolytic enzymopathies. However, this test is being used less frequently, because it is no more sensitive than the incubated osmotic fragility test, and also it is fairly time-consuming, such as the incubation at 37 ° for 48 h stage. Increased autohemolysis was observed in 92.8% of Japanese HS patients [7]. This abnormality was corrected by the addition of glucose or adenosine-5'-triphosphate.

10.3

Epidemiology and Genetics

Hereditary spherocytosis is the most common red cell membrane disorder in Northern European Caucasians [4–6], with a prevalence of roughly 1 in 5000. In the Japanese population, this is especially true in that HS is the most common disease among the congenital hemolytic anemias, because the gene for sickle cell anemia is not present in the Japanese population, and also the incidence of red cell glycolytic enzymopathies (especially glucose-6-phosphate dehydrogenase deficiency) is extremely low. Based on the nationwide survey study, which was carried out in 1974 at university hospitals and major hospitals specializing in hematology that were assigned to the Hemolytic Anemia Study Committee of the Ministry of Health and Welfare of the Japanese Government in 1974 [12], the incidence of congenital hemolytic anemias, of which HS accounted for 74.2%, was 5.7–20.3 per 10⁶ of the Japanese population, compared with 200–300 per 10⁶ in Caucasians.

However, very mild forms of this disorder may be much more common in Caucasians and in Japanese.

The relative incidence of HS among red cell membrane disorders of 1014 patients from 605 kindred in the Japanese population was 57.3% (581 cases from 303 kindred) [13]. In this study, most of the protein 4.2 anomalies (34 patients from 20 kindred) were incorporated into the category of HS, except for seven cases of two traits with doublet protein 4.2 anomalies, which demonstrated hereditary stomatocytosis. Therefore, HS appears to be most the common red cell membrane disorder in the Japanese population.

Regarding the genetic inheritance of HS, typically this disorder has been considered to be autosomal dominantly (AD) inherited. However, a nationwide survey by the Hemolytic Anemia Study Committee organized by the Ministry of Health and Welfare of the Japanese Government demonstrated that there were a substantial number of cases with HS in whom AD transmission was not shown (62% of the total kindred) by conventional examinations in clinical hematology [7, 12]. These HS cases of non-AD type are usually classified as “sporadic” cases. In this category, three possibilities for the pathogenesis can be considered, i.e., there are: (1) patients in whom HS occurred for the first time without hereditary inheritance (so-called “*de novo*” HS); (2) homozygotes by autosomal recessive (AR) inheritance, among whom heterozygotes of AR inheritance are usually asymptomatic and do not appear to be sick; and (3) cases of mild severity even with AD inheritance, in whom the establishment of heredity may be difficult. These three possibilities can be differentiated only by molecular biology analyses. In fact, a homozygous patient of band 3 Fukuoka with AR inheritance [14], and a number of patients with total protein 4.2 deficiencies due to protein 4.2 gene mutations [15] as homozygotes or compound heterozygotes have been identified in the Japanese population.

To determine the extent of clinical severity, complete family studies have been performed in 79 HS patients from the 60 kindred [16]. Among these patients, the HS of 35 from 19 kindred was because of AD inheritance. The remainder of the patients (44 cases: 56%) failed to demonstrate AD transmission. Therefore, these patients were classified as non-AD transmitted HS.

In 30 patients from 15 kindred of the AD-HS group, hemoglobin content before splenectomy was 12.3 ± 2.0 g dL⁻¹, mean corpuscular volume (MCV) 86.8 ± 5.6 fL, mean cell hemoglobin concentration (MCHC) 35.7 ± 1.1 %, reticulocytes 9.7 ± 5.8 %, and indirect bilirubin level 1.6 ± 0.8 mg dL⁻¹. In 41 unsplenectomized patients of the non-AD HS group, hemoglobin was 10.6 ± 1.8 g dL⁻¹, MCV 88.1 ± 4.9 fL, MCHC 34.9 ± 1.4 %, reticulocytes 11.9 ± 4.5 %, and indirect bilirubin level 2.4 ± 1.3 mg dL⁻¹. Therefore, contrary to the commonly-held belief, clinical HS of the AD type and that of the non-AD type were almost identical, both demonstrating moderate uncompensated hemolytic anemia with almost the same severity [16].

10.4

Pathogenesis: Affected Proteins and Their Related Gene Mutations

Quantitative abnormalities of several membrane proteins have been identified in red cells of patients with HS [4–6, 17]. The molecular basis of HS is heterogeneous. Based on a densitometric quantitation of membrane proteins separated by SDS–PAGE, HS can be categorized into the following subgroups: (1) combined partial deficiency of spectrin and ankyrin due to ankyrin gene mutations, (2) partial deficiency of band 3 protein due to band 3 gene mutations, (3) partial β -spectrin deficiency due to spectrin gene mutations, (4) total deficiency of protein 4.2 due to protein 4.2 gene mutations, and (5) other less common abnormalities.

In Western countries, combined partial spectrin and ankyrin deficiency is most commonly observed (in approximately 55 % of HS patients), followed by partial band 3 deficiency (approximately 27 %), protein 4.2 deficiency (roughly 3 %), and other abnormalities (about 15 %) including β -spectrin anomalies [10, 18, 19].

In Japanese HS patients with unrelated families who were studied by SDS–PAGE, there were fewer ankyrin deficiencies (7 %), a moderate number of band 3 deficiencies (20 %), and many more protein 4.2 deficiencies (45 %), with 28 % of unknown etiology.

Based on the abnormalities of membrane proteins, the mutations of these membrane protein-related genes have been identified. Since each kindred of HS has basically a unique mutation, there appears to be no selective advantage to mutations in spite of a few rare exceptions such as total deficiency of protein 4.2 [11, 13, 15], in which most of the affected cases are due to a homozygous missense mutation of the protein 4.2 gene of the Nippon type.

10.4.1

Combined Partial Deficiency of Spectrin and Ankyrin Due to Ankyrin Gene Mutations

Ankyrin is a large, 206 kDa, 8.3×10 nm protein that provides the primary linkage between the spectrin–actin based red cell membrane cytoskeleton and the lipid bilayer of the plasma membrane. This important cellular localization of membrane proteins may be mediated by the relative affinities of the many different isoforms of ankyrin for target proteins: membrane skeletal proteins, ion transport proteins, and cell-adhesion molecules. The isoform diversity of ankyrin arises from both different gene products and alternative splicing of the same gene product (see Sections 6.1 and 16.1).

Ankyrin 1, red cell ankyrin, has been identified in erythroid tissue, brain, and muscle. The major form of ankyrin 1 is composed of three domains: (1) an 89 kDa NH₂-terminal domain composed of 24 conserved repeats known as a membrane domain that contains the binding site for band 3; (2) a 62 kDa domain that contains the binding sites for spectrin and vimentin; and (3) a 55 kDa COOH-terminal regulatory domain (Fig. 6.1). Complex patterns of alternative splicings have been identified in the region encoding the regulatory domain (see Section 6.1).

In Western countries, a combined deficiency of spectrin and ankyrin is the most common feature in HS red cell membranes [5, 18, 20–31]. Ankyrin mutations in HS (Table 10.1) were first reported in German patients [20]. Frameshift mutations and nonsense mutations of the ankyrin gene were found mostly in HS patients of autosomal dominant (AD) inheritance, and missense mutations tended to be detected mostly in HS patients of non-AD transmission. Frameshift or nonsense mutations are believed to lead to a defective ankyrin molecule, ankyrin deficiency, or both. Missense mutations may disrupt normal ankyrin–protein interactions. A list of ankyrin gene mutations is shown in Table 10.1.

Table 10.1 Membrane protein gene mutations in hereditary spherocytosis.

I. Mutations of the red cell ankyrin 1 (Ank 1) gene in hereditary spherocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Promoter					
Unnamed	–	–204 C→G	–	Promoter	AD
Unnamed	–	–153 G→A	–	Promoter	AR
Unnamed	–	–108 T→C	–	Promoter	AR
Unnamed	–	–72/–73 2nt del (–TG)	–	Promoter	AR
Band 3-binding domain					
Chiba II	2–5	10 nt del: –CCCTATTCTG	PCT	Frameshift (Del)	AD
Nara II	5 nt after exon 1	27 + 5 G→C	Deletion spliced	Abnormal splicing	AD
Saitama	111 or 112	1nt del (–T)	PCT	Frameshift (Del)	S
Bugey	146	437 1 nt del (–C)	PCT	Frameshift (Del)	S
Osterholz	174	520–539 del (–20 nt)	PCT	Frameshift (Del)	S
Shiga	3–4 nt after exon 5	1 nt ins (+A)	PCT spliced	Abnormal splicing	S
Tokyo II	187–190	10 nt del: –CACGGCTGCG	PCT	Frameshift (Del)	S
Limeira	277	CAC → CGC	H277R	Missense	AD
Stuttgart	329	985–986 2 nt del (–GC)	PCT	Frameshift (Del)	AD
Bari	428	1282 1 nt del (–G)	PCT	Frameshift (Del)	S
Walsrode	463	GTC→ATC	V463I	Missense	AR
Florianopolis	507	1519–1520 1 nt ins (+C)	PCT	Frameshift (Ins)	AD
Laguna	535	1605 1 nt del (–A)	PCT	Frameshift (Del)	

I. Continued.

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Kyoto	5 nt after end of exon 8	AGgtggg→AGgtggc	Spliced	Abnormal splicing	AD
Tokyo III	571, 572 or 573	1 nt del (–C)	PCT	Frameshift (Del)	S
Napoli I	573	1718 1 nt del (–T)	PCT	Frameshift (Del)	S
Einbeck	573	1717–1718 1nt ins (+C)	PCT	Frameshift (Ins)	AD
Aichi	592–593	GGCGGC→GGCGGGGCGGC	PCT	Frameshift (Ins)	
Munster	596	1788 1nt del (–C)	PCT	Frameshift (Del)	AD
Duisberg	601	1801–18 C→A	Spliced	Abnormal splicing	AD
Osaka II	612	CAG→TAG	Q612X	Nonsense	S
Votice	631	GAG→TAG	E631X	Nonsense	AD
Osaka I	637	1 nt ins (+C)	PCT	Frameshift (Ins)	S
Olomouc	765	TCG→TAG	S765X	Nonsense	AD
Marburg	797	2389–2392 4 nt del (–TAGT)	PCT	Frameshift (Del)	AD
Kagoshima	798–799	4 nt del (–CAGT)	PCT	Frameshift (Del)	S
Yamagata	1 nt after exon 22	2461 + 1 GT→CT	Deletion spliced	Abnormal splicing	S
<i>Spectrin-binding domain</i>					
Tabor	907	2720 1 nt del (–G)	PCT	Frameshift (Del)	AD
Napoli II	933	2799 1 nt del (–C)	PCT	Frameshift (Del)	S
Benesov	941	2825–2826 5 nt ins	PCT	Frameshift (Ins)	AD
Mie	951–953	7 nt del (–GCCGCCT) and 4 nt ins (+TCTG)	PCT	Frameshift (Del and Ins)	S
Anzio	983	2948 2 nt del (–CA)	PCT	Frameshift (Del)	S
Nara I	1046	CTA→CCA	L1046P	Missense	
Melnik	1053	CGA→TGA	R1053X	Nonsense	AD
Jaguariúna	1054	ATC→ACC	I1054T	Missense	AD
Tubarao	1075	ATC→ACC	I1075T	Missense	
Porta Westfalica	1127	3380 1 nt del (–C)	PCT	Frameshift (Del)	S
Unnamed	1185	TGG→CGG	W1185R	Missense	
Chiba I	1 nt after exon 28	3327 + 1 GT→CT	Deletion spliced	Abnormal splicing	S

I. Continued.

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Chiba III	1230	TAC→TAG	Y1230X	Nonsense	S
Tokyo I	1252	CGA→TGA	R1252X	Nonsense	AD
Unnamed	1262	3785–3791 7 nt del	PCT	Frameshift (Del)	
Kalrupy	1382	4145 1 nt del (–T)	PCT	Frameshift (Del)	AD
Regulatory domain					
Bovenden	1436	CGA→TGA	R1436X	Nonsense	AD
Chiba IV	1437	GTG →TG	PCT	Frameshift (Del)	
Karlov	1488	CGA→TGA	R1488X	Nonsense	AD
Prague	1512	1512–1513 201 nt ins	67 amino acids inserted	Insertion	
Düsseldorf	1592	GAC→AAC	D1592N	Missense	AR
Okayama	1 nt before start of exon 37	agTG→aaTG	Spliced	Abnormal splicing	
Toyama	1640	CAG→TAG	Q1640X	Nonsense	AD
Rakovnik	1669	GAA→TAA	E1669X	Nonsense	AD
Unnamed	1700	5097–34 C→T	Spliced	Abnormal splicing	AD
Saint–Etienne 1	1721	TGG→TGA	W1721X	Nonsense	AD
Saint–Etienne 2	1833	CGA→TGA	R1833X	Nonsense	AD
Bocholt	1894	5619+16 C→T	Spliced	Abnormal splicing	AR

II. Mutations of the red cell band 3 (AE1) gene (*EPB3*) in hereditary spherocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Promoter					
Genas	–	62 nt before codon 1 G→A	Promoter	–	AR
Neapolis	–	2 nt after exon 2 T→C	Loss of initiation	Abnormal splicing	AD
Cytoplasmic domain					
Montefiore	40	GAG→AAG	E40K	Missense	AR ?
Foggia	55	163 1 nt del (–C)	PCT	Frameshift (Del)	AD
Kagoshima	56	167 1 nt del (AAG→AG)	PCT	Frameshift (Del)	S

II. Continued.

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Hodouin	81	TGG→TGA	W81X	Nonsense	
Bohain	81	241 1 nt del (–T)	PCT	Frameshift (Del)	AD
Capetown	90	GAG→AAG	E90K	Missense	AR ?
Napoli I	100	298–299 1 nt ins (+T)	PCT	Frameshift (Ins)	AD
Fukuyama I	112–113 336–337	2 nt del (–AG or –GA)	PCT	Frameshift (Del)	AD
Nachod	117–121	3 nt before exon 6 C→A	5 amino acids (GTVLL) deleted	Abnormal splicing	AD
Fukuoka	130	GGA→AGA	G130R	Missense	AR
Montego	147	CCT→TCT GAG→AAG	P147S E40K	Missense	AD
Osnabrück I	150	CGA→TGA	R150X	Nonsense	AD
Lyon	150	CGA→TGA	R150X	Nonsense	AD
Wilson	172	515 1 nt del (–G)	PCT	Frameshift (Del)	AD
Worcester	172	515–516 1 nt ins (+G)	PCT	Frameshift (Ins)	AD
Fukuyama II	183	1 nt ins (+ A) (GAT→GAAT)	PCT	Frameshift (Ins)	AD
Campinas	204	694+1 G→T	Spliced	Abnormal splicing	AD
Princeton	275	823–824 1 nt ins (+C)	PCT	Frameshift (Ins)	S
Okayama	276	1 nt del (–C)	PCT	Frameshift (Del)	S
Boston	285	GCT→GAT	A285D	Missense	AD
Tuscaloosa	327	CCC→CGC	P327R	Missense	AR ?
Noirterre	330	CAG→TAG	Q330X	Nonsense	AD
Transmembrane domain					
Brüggen	419	1255 1 nt del (–C)	PCT	Frameshift (Del)	AD
Benesov	455	GGG→GAG	G455E	Missense	AD
Yamagata	455	GGG→AGG	G455R	Missense	S
Bicêtre II	456	1366 1 nt del (–G)	PCT	Frameshift (Del)	AD
Pribram	478	1431+1 G→A	Spliced	Abnormal splicing	AD
Coimbra	488	GTG→ATG	V488M	Missense	AD

II. Continued.

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Bicêtre I	490	CGC→TGC	R490C	Missense	AD
Evry	496	1486 1 nt del (–T)	PCT	Frameshift (Del)	AD
Milano	500	1498–1499 69 nt ins	23 amino acids inserted	Duplication	AD
Dresden	518	CGC→TGC	R518C	Missense	AD
Chiba	526 or 527	1 nt del (–C)	PCT	Frameshift (Del)	S
Smichov	616	1848 1 nt del (–C)	PCT	Frameshift (Del)	AD
Trutnov	628	TAC→TAA	Y628X	Nonsense	AD
Hobart	647	1940 1 nt del (–G)	PCT	Frameshift (Del)	S
Osnabrück II	664	3 nt del (–ATG)	M664 deleted	Deletion	AD
Most	707	CTG→CCG	L707P	Missense	AD
Okinawa	714	GGG→AGG	G714R	Missense	AD (Compound)
Prague II	760	CGG→CAG	R760Q	Missense	AD
Kumamoto	760	CGG→CAG	R760Q	Missense	S
Hradec Kralove	760	CGG→TGG	R760W	Missense	AD
Tochigi I	760	CGG→TGG	R760W	Missense	S
Tochigi II	760	2058–5 1 nt del (–A)	Spliced	Abnormal splicing	S
Chur	771	GGC→GAC	G771D	Missense	AD
Napoli II	783	ATC→AAC	I783N	Missense	AD
Jablonec	808	CGC→TGC	R808C	Missense	AD
Nara	808	CGC→CAC	R808H	Missense	S (de novo)
Prague I	822	2464–2465 10 nt ins	PCT	Frameshift (Ins)	AD
Birmingham	834	CAC→CCC	H834P	Missense	AD
Nagoya	837	ACG→AGG	T837R	Missense	AD
Philadelphia	837	ACG→ATG	T837M	Missense	AD
Tokyo	837	ACG→GCG	T837A	Missense	S
Prague III	870	CGG→TGG	R870W	Missense	AD
Vesuvio	894	2682 1 nt del (–C)	PCT	Frameshift (Del)	AD

III. Mutations of the red cell protein 4.2 gene (*ELB42*) in hereditary spherocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Lisboa	89	264 or 265 1 nt del (–G) (AAGGTG→AAGTGG)	PCT	Frameshift (Del)	AR
Fukuoka	119	357 TGG→TGA	W119X	Nonsense	AR
Nippon	142	424 GCT→ACT	A142T	Missense	AR
Komatsu	175	523 GAT→TAT	D175Y	Missense	AR
Notame	308	922+1 G→A	Spliced	Abnormal splicing	AR
Tozeur	310	929 CGA→CAA	R310Q	Missense	AR
Shiga	317	949 CGC→TGC	R317C	Missense	AR
Nancy	317	950 CGC→CC	PCT	Frameshift	AR

PCT: premature chain termination

IV. Mutations of the red cell β -spectrin gene (*SPTB*) in hereditary spherocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
<i>Actin/P4.1 binding domain</i>					
Promissão	1	1 ATG→GTG	M1V (no protein)	No translation	AD
Guemene-Penfao	100	300 G→C	Spliced	Abnormal splicing	AD
Atlanta	182	TGG→GGG	W182G	Missense	AD
Unnamed	189	GGC→GAC	G189A	Missense	AD
Kissimmee	202	TGG→CGG	W202R	Missense	AD
Ostrava	202	604 del 1 nt (–T)	PCT	Frameshift (Del)	AD
Oakland	220	ATC→GTC	I220V	Missense	AD
<i>Spectrin repeats</i>					
Bicêtre	444–446	1331–1338 8 nt del	PCT	Frameshift (Del)	AD
Alger	514	CAG→TAG	Q514X	Nonsense	AD
Philadelphia	590	1767–1768 1 nt ins (+A)	PCT	Frameshift (Ins)	AD
St. Barbara	638	1912 1 nt del (–C)	PCT	Frameshift (Del)	S
Bergen	783–784	2351–2352 1 nt ins (+A)	PCT	Frameshift (Ins)	AD
Baltimore	845	CAG→TAG	Q845X	Nonsense	AD

IV. Continued.

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
Houston	926	2777 1 nt del (–A)	PCT	Frameshift (Del)	AD
Winston-Salem	936–1255	3764+1 G→A	Spliced	Abnormal splicing	S
Columbus	1227	CCT→TCT	P1227S	Missense	AD
Durham	1492–1614	Exons 22 and 23 deleted	L1492–K1614 del	Deletion	S
Birmingham	1684	CGC→TGC	R1684C	Missense	AR
Sao Paulo	1884	GCG→GTG	A1884V	Missense	S
Tabor	1946	CAG→TAG	Q1946X	Nonsense	AD

V. Mutations of the red cell α -spectrin gene (SPTA) in hereditary spherocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>	<i>Inheritance</i>
<i>Spectrin repeats</i>					
Bughill	970	GCT→GAT	A970D	Missense	
LEPRA	1449	4339–99 C→T	Spliced	Abnormal splicing	AR
Prague	1730	5187–2 A→G	Spliced	Abnormal splicing	AR

In the Japanese HS patients, 16 out of 49 patients were found to carry ankyrin gene mutations in heterozygous states [21]. The mutations pathognomonic for HS consisted of four nonsense mutations, eight frameshift mutations, and four abnormal splicing mutations (33 % of totally unrelated HS) (Table 16.1). It is interesting to note that four mutations were detected in 16 unrelated patients of AD HS (25 %) and that 12 mutations were found in 30 sporadic HS patients (40 %). Thus, ankyrin gene mutations are not rare in the Japanese population (probably one-third of all HS patients of AD and non-AD inheritance), although the frequency of the mutations is not as high as that in HS patients in Western countries, where ankyrin gene mutations are found in 55–60 % of all HS patients [18, 19].

The ankyrin gene mutations appear to confer more clinically severe effects than the band 3 gene mutations. The reason is probably that the ankyrin gene mutations were mainly more serious mutations (four nonsense mutations, seven frameshift mutations, and four splicing mutations) compared with the milder mutations (four frameshift mutations but eight missense mutations) of the band 3 gene mutations. In fact, the hemoglobin levels and the percentage of reticulocytes in the HS patients with ankyrin gene mutations and in those with band 3 gene mutations

were 9.0 ± 2.3 and 12.7 ± 1.7 g dL⁻¹, and 15.8 ± 7.4 and 8.4 ± 1.2 %, respectively [21]. Therefore, the HS patients with ankyrin gene mutations were more severely anemic with markedly increased reticulocytosis than those with band 3 gene mutations.

Regarding the red cell membrane protein contents in the Japanese HS patients studied, the spectrin levels ranged from -8.7 % to 4.6 % (-1.9 ± 3.9 %), protein 4.2 from -28.1 % to -2.4 % (-12.1 ± 8.0 %), and ankyrin from -8.3 % to 19.7 % (4.4 ± 7.9 %) of that of normal subjects [21]. Therefore, the protein 4.2 contents were consistently lower in these patients. In contrast, the ankyrin content did not appear to be decreased, probably for several reasons, including increased reticulocytosis leading to increased ankyrin content, which may compensate for possible ankyrin deficiency, and the methodological limitations of the SDS-PAGE [21]. In contrast, the band 3 content was decreased (17 %) consistently in 30 patients with non-ankyrin gene mutations, which included the patients with band 3 gene mutations [16, 21].

It is interesting to note that frameshift mutations were found from exon 1 to exon 26, especially in the band 3 binding domain of ankyrin 1, contrary to the non-sense mutations that were present mostly at the 3'-terminal side, particularly in the spectrin-binding domain and the regulatory domain [4, 5, 21]. It should also be noted that all of the 16 mutations pathognomonic for HS have recently been published, and are limited to the Japanese population [21].

Two polymorphic missense mutations and 15 silent mutations were also found in the Japanese HS patients [16, 21] (Table 16.2). No significant difference was observed in the allele frequency of these gene polymorphisms between normal subjects and HS patients [16, 21]. These polymorphisms were observed to almost the same extent in Japanese and German populations [16].

An interesting case of HS is ankyrin Walsrode, in which red cell membranes were deficient in band 3 as well as ankyrin and spectrin, is due to a mutation in the band 3 binding domain of ankyrin that has a decreased affinity for band 3 [20].

Mutations on the promoter region of the ankyrin gene have also been identified in the HS patients [18, 22].

Pathogenesis of a combined deficiency of spectrin and ankyrin lies in the ankyrin gene mutations [18, 20–31], since ankyrin represents the major binding site for spectrin on the membrane (see Section 6.1). Therefore, ankyrin deficiency is accompanied by a proportional decrease in the spectrin assembly on the membrane despite normal spectrin synthesis. Most ankyrin mutations are frequently associated with decreased mRNA accumulation leading to decreased ankyrin content. Even amongst HS affected members of the same kindred with the ankyrin gene mutation, clinical severity varies because of parental mosaicism for the ankyrin mutation.

The important role of the ankyrin gene in the pathogenesis of HS was initially discovered in HS patients with an absence of the entire ankyrin gene on the short arm of chromosome 8 due to a large interstitial deletion [23, 32–38]. In these patients, there were various karyotypic abnormalities involving deletions or translocations of the ankyrin gene locus (8p11.2), such as deletions of 8p11.1 [32],

8p11.22–8p21.1 [33], 8p11–8p21.1 [23], and 8p11.23–8p21.1 [34], and translocations of t (8; 12) (p11; p13) [35, 36], and t (3; 8) (p21; p22) [37]. Glutathione reductase deficiency is also reported in some of these HS cases due to a deletion of its chromosomal gene locus (8p21.1) [38].

Ankyrin deficiency was also reported in a mouse strain (nb/nb) [39], in which ANK_R was missing in red cells and cerebellar Purkinje cells despite normal expression of ANK_B in the Ranvier nodes. The number of cerebellar Purkinje cells, which was normal at birth, decreases to 50 % of the normal level 6–9 months after birth, developing tremor and cerebellar symptoms [40].

Abnormalities in membrane protein genes have been detected, and their genotypes identified. The next stage should be to determine whether or not these genetic mutations are actually pathognomonic for HS. Direct evidence is required to clarify the pathognomonic role of these genetic mutations for the determination of phenotypes. For this purpose, electron microscopic studies of the red cell membranes *in situ* are most suitable for detecting the phenotypic abnormalities in detail (see Chapter 3). Electron microscopy (EM) with the quick-freeze deep-etching (QFDE) method or by the surface replica (SR) method was utilized to examine the cytoskeletal network (see Section 3.2.2). The number and size of the basic cytoskeletal units evaluated the condition of the cytoskeletal network *in situ*. EM with the freeze fracture (FF) method is adequate for evaluation of the condition of the integral proteins (see Section 3.2.3), especially band 3 as intramembrane particles (IMPs). The number, size, and distribution pattern of the IMPs are valuable indices for evaluating the conditions of band 3 molecules.

Two ankyrin gene mutations in the German Caucasian HS patients, ankyrin Marburg and ankyrin Stuttgart, were selected for this evaluation [41]. Both were associated with frameshift mutations at exon 22 in ankyrin Marburg or at exon 10 in ankyrin Stuttgart [20]. In these patients, mRNA was not detected on the mutated alleles, leading to decreased ankyrin content (approximately 70 % of normal), which was expressed through the normal ankyrin allele as a heterozygote.

Under these conditions, the number of cytoskeletal units was reduced by 30 %, as reflected by the decreased content of normal ankyrin molecules. The regular small size of the cytoskeletal units was significantly decreased concomitant to the increased larger size of these units, implying that the cytoskeletal network was not formed properly because of the disruption of the network [41]. The localization of ankyrin molecules on the red cell membranes *in situ* was markedly deranged, indicating that the availability of epitopes of ankyrin molecules against the anti-ankyrin antibody was extremely low compared with the protein content of ankyrin [41]. This result strongly suggests that conformational change in ankyrin molecules is present with the frameshift mutations in ankyrin Marburg and ankyrin Stuttgart. It is interesting to note that the number and size of IMPs were not affected in these ankyrin mutations. The results are shown in detail in Section 16.1.3 (Figs. 16.1–16.5).

10.4.2

Partial Deficiency of Band 3 Due to the Band 3 Gene Mutations

As described previously, the incidence of band 3 deficiency in red cells of Caucasian HS patients has been reported to be up to 30% (Table 10.1) [18, 19]. In Japanese HS patients, the incidence of band 3 deficiency was also around 20–30% [13].

To date, 55 band 3 gene mutations [5, 9, 16, 19, 20, 42–57, 60–63] have been reported worldwide (Table 10.1), including 27 missense mutations, 23 frameshift mutations and nonsense mutations, one splicing abnormality, two nucleotide duplications, one in-frame deletion, and one nucleotide substitution at the promoter region [4, 5]. The missense mutations are predominant, making up 50% of the total band 3 mutations. The band 3 mutations in the coding regions are clustered at exons 4 and 5, 9 and 10, and 17–19, which appear to be so-called “hot spots”, although band 3 gene mutations are spread throughout band 3, occurring both in the cytoplasmic domain as well as in the membrane domain (Fig. 1.5). The missense mutations frequently tend to be localized at the region corresponding to the membrane domain at the 3' end, especially at the cluster R region (codon 760 at exon 17 and codon 808 at exon 18) (Fig. 5.1). The frameshift and nonsense mutations are densely localized at the region corresponding to the cytoplasmic domain of the band 3 molecule at the 5' end.

Among these reported band 3 mutations, 12 pathognomonic mutations of the band 3 gene have been detected in 79 Japanese HS patients from 60 kindred; i. e., three frameshift mutations, eight missense mutations, and a splicing abnormality [16]. The mode of inheritance for these mutations was autosomal dominant (AD) inheritance in five kindred, autosomal recessive (AR) inheritance in one kindred, *de novo* in one patient, and sporadic in five patients. In a kindred with AR inheritance, the patients were homozygotes of a missense mutation (band 3 Fukuoka) [14] (see Section 15.1.4). Under these conditions, their parents were asymptomatic as heterozygotes.

The kindred with band 3 Okinawa was unique with respect to the genotype [42] (see Section 15.1.5). In this kindred, four different types of band 3 gene mutations were detected: allele Fukuoka (G130R: GGA→AGA) and allele Okinawa (K56E: AAG→GAG, P854L: CCG→CTG, and G714R: GGG→AGG) *in trans*. In this proband, protein 4.2 was missing completely, disproportionately to the moderately decreased band 3 content. As the level of detection by single strand conformational polymorphism (SSCP) is approximately 70%, every patient with a reduced amount of band 3 appears to have a mutation in the band 3 gene. Furthermore, it should be noted that, in these band 3 mutations, the protein 4.2 content usually decreased in proportion to the extent of decrement of band 3 content, although no abnormality was detected in the protein 4.2 gene *per se*. The allele Fukuoka is known to alter the binding of protein 4.2 to band 3 [14]. The proband (the daughter) presented with a pronounced decrease of band 3 ($49.8 \pm 0.3\%$ of normal), and established an almost complete lack of protein 4.2 with only traces (less than 0.1% of normal) of 72, 68 and 66 kDa fragments of protein 4.2. Her mother showed partial deficiency in band 3 (–25% on average) and a proportional reduction in protein

4.2. Therefore, the mother was heterozygous for an allele of the EPB3 gene, allele Okinawa, and her daughter was a compound heterozygote of allele Okinawa and allele Fukuoka [42]. Heterozygosity for allele Fukuoka has been documented in another individual who showed no clinical or hematological signs, and normal band 3 content [14]. It has been suggested that band 3 Okinawa binds virtually all the protein 4.2 in red cell precursors, band 3 Fukuoka being unable to do so, and that band 3 Okinawa cannot be incorporated into the membrane leading to degradation of the band 3 Okinawa protein complex. In contrast, band 3 Fukuoka, free from bound protein 4.2, could then be incorporated normally into the lipid bilayer. Thus, it has been speculated that protein 4.2 would not appear in the proband's red cell membranes.

Partial deficiency of protein 4.2 is fairly common in HS with band 3 mutations [9, 19, 20, 42–62] under the following two situations: (1) partial or total lack of one haploid set of mutated band 3, and (2) mutations in the cytoplasmic domain of band 3, which contains major binding sites for protein 4.2. These conditions will be discussed in a separate section (Section 16.2).

Band 3 deficient Caucasian HS patients may present a small number of mushroom-shaped or “pincered” red cells on peripheral blood smears [8, 9], although this finding has not been observed in the Japanese HS patients.

Alleles have been detected that influence band 3 expression and that aggravate band 3 deficiency and worsen the clinical severity of disease, when inherited *in trans* to a band 3 mutation (band 3 Genas and band 3 Lyon) [53, 63]. In some HS patients with band 3 deficiency, band 3 gene expression may be reduced or a band 3 mutation may interfere with the proper co-translational insertion of band 3 into membranes of the endoplasmic reticulum or translocation of band 3 to the plasma membrane.

In some HS patients, a number of band 3 mutations clustered in the membrane domain, where highly conserved arginines are replaced as a result of band 3 gene mutations [43]. These arginines are located at the cytoplasmic end of a transmembrane helix, and appear to aid in maintaining the orientation of the transmembrane domain. Therefore, the mutated band 3 appears neither to fold nor to insert into the endoplasmic reticulum and finally into the red cell membrane after synthesis. These short inframe deletions by frameshift mutations or nonsense mutations and missense mutations of the band 3 gene, especially in its transmembrane domain, should impair normal trafficking and folding of band 3 molecules and should inhibit the insertion of these molecules into the membrane [102].

Complete deficiency of band 3 has been reported in humans [52, 62, 63], cows [58], and mice [59, 64]. In the human case, lethal and near lethal HS associated with hydrops fetalis, metabolic acidosis, and severe anemia with complete deficiency of band 3 and protein 4.2 from red cell membranes have been described in members of a large Portuguese kindred homozygous for a band 3 mutation (band 3 Coimbra: V488M) [62, 63]. There was a couple whose members carried the mutation Coimbra in the heterozygous state. At the second pregnancy of this couple, homozygosity for mutation Coimbra was ascertained antenatally and the pregnancy was interrupted. At the third pregnancy, a severely anemic hydropic

female baby in the homozygous state was reanimated and kept alive with an intensive transfusional regimen. Cord blood smears disclosed dramatic erythroblastosis and poikilocytosis. Red cells with a tail-like elongation were a conspicuous feature. Band 3 and protein 4.2 were completely absent in red cell membranes. Metabolic acidosis and nephrocalcinosis were present. The total absence of band 3 in humans appears to be reasonably compatible with life as long as intensive transfusion support is provided. In the heterozygous state, the band 3 content, 4,4'-diisothiocyanato 1,2-diphenylethane-2,2'-disulfonate (H_2 DIDS) sites ($\mu\text{mol L}^{-1}$), and sulfate flux ($\text{nmol } 10^{-8}$ red cells per 10 min) were on average -23% of normal, -35% of normal, and -34% of normal, respectively.

The second example of total band 3 deficiency is Japanese cattle due to a nonsense mutation of the band 3 gene [58]. A moderate anemia of autosomal incompletely dominant inheritance with marked microspherocytosis has been reported in Japanese cattle. In these cattle, no band 3 was detected due to a nonsense mutation (CGA→TGA; Arg→stop) of the band 3 gene at the position corresponding to codon 646 in human red cell band 3 cDNA. Protein 4.2 was almost completely absent. Considerable decreases were also observed in other major red cell membrane components such as spectrin, actin, glyceraldehyde 3-phosphate dehydrogenase (band 6), and ankyrin (a reduction by at least 50% of normal subjects). The probands also exhibited a marked distortion and disruption of the membrane skeletal network with tremendous instability. The affected cattle also lacked kidney band 3. The proband's red cells completely lacked rapid anion exchange as a function of band 3 protein; i.e., the defective $\text{Cl}^-/\text{HCO}_3^-$ exchange in these cells was uncompensated for and limited to a rather low level. This was not lethal to the affected cattle as is observed in human cases, even though the body weight was substantially lower than normal at the same age. The results are shown in detail in Section 15.1.3 (Figs. 15.1–15.5).

The third example is knock-out mice by targeted disruption of the band 3 gene [64], or those by selectively targeted inactivation of the erythroid band 3 gene [59], in which kidney band 3 was not affected. Targeted disruption of the murine band 3 gene has resulted in spherocytosis and severe hemolytic anemia. Erythroid band 3 gene was selectively inactivated but not the kidney band 3 gene [64]. Red cells of homozygous mice were completely devoid of band 3 protein, whereas normal levels of band 3 protein were detected in the lysates of kidneys obtained from band 3^{-/-} mice. The mutant red cell membrane ghosts contained 75% of the normal spectrin, significantly reduced ankyrin (40% of normal), and no detectable protein 4.2. Normal amounts of protein 4.1 and actin were detected in homozygous mice. The presence of a reduced but significant amount of ankyrin in band 3^{-/-} ghosts gives further support to the existence of band 3-independent sites for the attachment of ankyrin in the red cell membrane. The concurrent loss of protein 4.2 in band 3^{-/-} red cells shows that the binding of protein 4.2 to the plasma membrane is determined exclusively by its interaction with band 3. The red cell phenotype of the mice of this type is consistent with the results obtained from the cattle with the homozygous nonsense mutation of the band 3 gene. The band 3^{-/-} red cells also contained adducin, dematin, p55, and glycophorin C. In contrast, the

band 3^{-/-} red cells are completely devoid of glycophorin A (GPA) despite the presence of normal GPA mRNA.

The function of band 3 was also examined in mice with targeted mutagenesis [59]. The mouse band 3 gene consists of 20 exons. A 1130 base pair segment between exons 9 and 11 was replaced with a neoR cassette. This segment encompasses the distal portion of the N-terminal cytoplasmic domain and the first membrane-spanning segment of the C-terminal domain. In the homozygous targeted mice, no band 3 transcript was detected in newborn reticulocyte or 14.5 day fetal liver RNA using a full-length band 3 cDNA. The absence of the normal band 3 gene product as well as the absence of any truncated band 3 polypeptides derived from the targeted gene was confirmed. Band 3^{-/-} red cell ghosts contained $84.7 \pm 5.5\%$, $86.4 \pm 5.4\%$, and $48.8 \pm 5.0\%$ of wild type, steady state levels of α -spectrin, β -spectrin, and ankyrin, respectively. In heterozygous state (band 3^{+/-}), normal amounts of α - and β -spectrin ($94.8\% \pm 5.4\%$ and $96.8 \pm 4.2\%$, respectively) and ankyrin ($119 \pm 6.9\%$) but decreased levels of band 3 ($82.3 \pm 2.1\%$) were observed.

Glycosylation may be affected in band 3 molecules of HS patients [65]. Three HS kindred with rapidly migrating band 3 due to a post-translational defect of band 3 glycosylation have been reported. Concomitant with this anomaly, the glycosylation of glycophorin A was also affected, resulting a more rapidly migrating glycophorin A on SDS-PAGE gels.

It has recently been reported that the expression of band 3 may be closely associated with that of glycophorin A [65, 66]. In a Japanese kindred (glycophorin A Hiroshima) [66], band 3 content in red cells was substantially decreased with impaired anion (phosphoenol pyruvate) transport. However, there was no mutation in the band 3 gene *per se*. Instead, a missense mutation (L75I) of the glycophorin A gene was detected as a heterozygote. The amount of glycophorin A, which was substantially decreased, corresponded to that of band 3 in this heterozygous patient. Therefore, the expressions of band 3 and glycophorin A appears to be closely associated with each other [66]. The same observation was made in the knock-out mice on the band 3 gene, in which no glycophorin A was detected [59].

In some HS patients with the band 3 gene mutations, the association of renal tubular acidosis has been reported (band 3 Pribram [56, 57], band 3 Campinas and, band 3 Coimbra [62]). Band 3 missense mutations have been found also in patients with dominant renal tubular acidosis of the distal type, although no red cell abnormalities have been reported in these patients [57, 60, 61]. Therefore, the exact mechanism of this pathogenesis has not been clarified.

Regarding the relationship between the genotype and the phenotype of band 3, the mutations of the band 3 gene are basically associated with HS as a phenotype. However, mutation of the deletion at codon 400–408, which corresponds to the region of the junction between the cytoplasmic domain and intramembrane domain, was detected in patients with Southeast Asian ovalocytosis (SAO), which is essentially a group of HE. This phenotypic difference with the mutation on the same band 3 gene is difficult to explain.

Numerous polymorphic mutations of the band 3 gene have been identified [4, 5, 16]. Among them, seven types of polymorphic mutations have also been detected

in 42 normal Japanese individuals and in 55 Japanese HS patients [16]. In addition to previously-reported polymorphic mutations (band 3 Darmstadt: D38A: GAC→GCC at exon 4; band 3 Memphis I: K56E: AAG→GAG at exon 4; a silent mutation: S438S: TCG→TCA at exon 12; and band 3 Diego: P854L: CCG→CTG at exon 19), there have been three new polymorphisms which have never before been reported, i. e., (1) band 3 Okayama (GAG→GAT; E72D in exon 5), (2) substitution A→G, at the 87th nucleotide after the end of exon 7, and (3) the deletion of three nucleotides (GAG), at the 30–32 nucleotides before the start of exon 8. There were no differences in allele frequency of these polymorphic mutations of the band 3 gene between the HS patients with pathognomonic band 3 mutations (16 alleles) and those without these band 3 mutations (92 alleles) [16].

As for the role of band 3 for the pathogenesis of HS, homozygosity for a band 3 defect causes a severe, but nonlethal hemolytic anemia [52, 58, 59, 62–64], and band 3 is critical for stabilizing the membrane skeleton via its interactions with ankyrin and protein 4.2.

To evaluate whether or not the mutations of the band 3 gene are pathognomonic for HS, electron microscopic studies of the red cell membranes *in situ* were carried out.

A homozygous patient was selected having the missense mutation of the band 3 gene (codon 130 GGA→AGA: Gly→Arg in exon 6) [14]. Therefore, all the band 3 molecules present in the red cell membrane *in situ* were composed of abnormal and mutated protein. Band 3 content in the proband was slightly decreased (90 % of normal), and a binding assay of the patients band 3 to normal ankyrin or protein 4.2 was markedly impaired. Therefore, the patient's protein 4.2 content was also markedly decreased (approximately 55 % of normal) disproportional to the nearly normal level of band 3, because of this impaired binding activity of band 3 with protein 4.2. As a pathogenesis of HS, this disorder appears to be derived from significantly decreased protein 4.2 content because of the mutated band 3, the mRNA of which was expressed. EM studies using the QFDE method revealed a slightly decreased number of IMPs (90 % of normal), with a slightly increased number of larger sized IMPs (partly oligomerized). These observations suggest that the effect of the missense mutation on the structural abnormality of red cell membranes may be minimal, even in a homozygous state [14] (see Section 15.1.4, Figs. 15.6–15.8).

Abnormality of the red cell membrane structure *in situ* was also examined in the red cells of the patient with autosomal dominantly inherited HS, in which the frameshift mutation (the deletion of A at codon 56 in exon 4) of the band 3 gene was established, band 3 Kagoshima [16]. In this patient, only one normal allele was expressed, but the mutated allele was not expressed. Therefore, band 3 content in the red cells was reduced to 70 % of normal. Reflecting this biochemical observation, the number of IMPs was reduced by 30 % but with normal size distribution compared with that in normal red cells. In contrast, the cytoskeletal network was not affected.

Total band 3 deficiency was found in Japanese bovine red cells, which were in a homozygous state of the nonsense mutation of the band 3 gene (codon 646

CGA→stop codon) [58]. Band 3 is completely missing in the red cells. The peripheral red cells demonstrated extremely marked microspherocytosis, with budding formation at the red cell surface. The cytoskeletal network was markedly disrupted, with endocytosis and exocytosis [58]. These findings clearly indicate the extreme instability of the cytoskeletal network in a condition where no band 3 molecules were present (see Section 15.1.3, Figs. 15.9–15.14).

10.4.3

Protein 4.2 Deficiency

Protein 4.2 abnormalities of congenital origin are classified into two groups [15, 67], i. e., (1) deficiency (reduced content) of protein 4.2, and (2) protein 4.2 variants with normal or nearly normal protein 4.2 content. The latter is associated with hereditary stomatocytosis. The first category of protein 4.2 deficiencies is further divided into two subgroups, i. e., (1) complete deficiency, and (2) partial deficiency [15, 67] (Fig. 16.6).

Complete deficiency of protein 4.2 is believed to be pathognomonic for HS or the disease state alike (see Section 16.2.2). Complete protein 4.2 deficiency is due to the mutations of the protein 4.2 gene *per se*, except for the HS family with band 3 Okinawa (Section 15.1.5), which were described previously. Partial protein 4.2 deficiency is fairly common in HS with band 3 gene mutations under the two following situations, i. e., (a) partial or total lack of one haploid set of mutated band 3, and (b) mutations in the cytoplasmic domain of band 3, which contains major binding sites for protein 4.2 [15] (Section 16.2.3). In the former situation, the extent of the decrease of protein 4.2 content was basically proportional to that of the band 3 content. In the latter situation, the decrement of the protein 4.2 content is disproportionately greater when mutations of the band 3 gene are present in its cytoplasmic domain, where the binding site(s) for protein 4.2 is located; e. g., band 3 Tuscaloosa (P327R: CCC→CGC) [51], band 3 Montefiore (E40K: GAG→AAG) [50], and band 3 Fukuoka (G130R: GGA→AGA) [14], as discussed previously. In these cases, protein 4.2 is sharply decreased due to the mutations on the band 3 gene.

Eight types of total protein 4.2 deficiency have been reported [15] (Table 10.1). The mutations of the protein 4.2 gene pathognomonic for this disease state have also been identified; i. e., four missense mutations, two frameshift mutations, one nonsense mutation, and one donor site mutation because of intronic substitution (Table 16.3). Therefore, missense mutations are predominant, especially allele protein 4.2 Nippon (142 GCT→ACT: Ala→Thr) [68], which has been observed in 17 patients of 13 families, among 28 patients of 19 kindred with complete protein 4.2 deficiency [13, 15]. These protein 4.2 gene mutations have been found mostly in the Japanese population; i. e., protein 4.2 Nippon, protein 4.2 Shiga [67, 69], protein 4.2 Komatsu [70], protein 4.2 Fukuoka [71], and protein 4.2 Notame [72]. Only three have been observed in the non-Japanese population; i. e., protein 4.2 Tozeur in Tunisia [73], protein 4.2 Lisboa in Portugal [74], and protein 4.2 Nancy in France [75].

Among these protein 4.2 gene mutations, the mutation of the Nippon type is most important in the Japanese population because this mutation is involved in homozygotes of the protein 4.2 Nippon and also in compound heterozygotes of protein 4.2 Shiga, protein 4.2 Fukuoka, and protein 4.2 Notame (Table 16.3).

Complete protein 4.2 deficiency due to the mutations of the protein 4.2 gene appears to be transmitted by autosomal recessive (AR) inheritance, and most patients have been homozygotes or compound heterozygotes of missense mutations on the protein 4.2 gene [15] (Table 16.3). Therefore, sole heterozygotes of these missense mutations have been asymptomatic, with nearly normal protein 4.2 content in red cells.

The morphological feature of red cells completely deficient in protein 4.2 was ovalostomatocytosis in protein 4.2 Nippon and in protein Komatsu [70], i.e., a mixed population of ovalocytosis and stomatocytosis with minimal microspherocytosis (Fig. 16.7). In contrast, in other complete protein 4.2 deficiencies, microspherocytosis was a predominant feature, resembling HS. Therefore, it remains controversial whether or not protein 4.2 Nippon can be categorized as classical HS [15].

Total deficiency of protein 4.2 is important to enable a clearer understanding of the pathogenesis of HS in the Japanese population when no positive family history is available, because the proband will be a homozygote of the missense mutations of the protein 4.2 gene mutation, and the parents as heterozygotes should be asymptomatic.

Although a slight variation in clinical observations exists among the reported cases with total protein 4.2 deficiency, the characteristic features are moderate, uncompensated hemolysis with moderate reticulocytosis and increased indirect bilirubin [15]. The hemolysis usually responds to splenectomy [15, 67]. In comparison with typical cases of classical, autosomal dominantly inherited HS, in which substantial microspherocytosis and increased mean corpuscular hemoglobin concentration (MCHC) are usual, the MCHC ($34.8 \pm 0.1\%$) is only minimally elevated in this disorder, reflected by minimal microspherocytosis [15].

Protein 4.2 content was completely or almost completely missing in the red cells of these patients. In the Nippon type, trace amounts of the 72 and 74 kDa peptides were detected by immunoblotting [15]. The 74 kDa peptide was not detected in protein 4.2 Komatsu without the missense mutation of codon 142 GCT→ACT [11, 70]. The content of band 3 was reduced by 10–20% of the normal value [15]. The contents of most other membrane proteins, including spectrin, ankyrin, and protein 4.1 were essentially unaffected. It has recently been reported that glycophorin C is substantially reduced in red cells with complete protein 4.2 deficiency.

The membrane deformability of protein 4.2 deficient red cells is distinctly abnormal, especially under conditions with various stresses such as heat treatment [15] (Fig. 16.9). The extent of the abnormality in the protein 4.2 deficiency was strikingly different from that in classical HS [15], in which no essential changes were observed under the same conditions, as was the case with normal controls. Red cell deformability is chiefly dependent on the functions of the cytoskeletons (see Section 2.3.4.2), which are mainly composed of spectrin in addition to protein

4.1 and actin. The cytoskeletal network appears to be linked to the lipid bilayer mostly via band 3 molecules in the presence of the normal amount of protein 4.2, which has been proven to be bound directly to spectrin molecules (see Section 6.2.2.1, Interactions of protein 4.2 with spectrin). Once these red cells are subjected to physicochemical stress, the network disassembles easily by losing its connection with the lipid bilayer.

Biophysical characteristics in protein 4.2 deficient red cells demonstrated that the extractability of band 3 was enhanced significantly up to 60% of the normal control, indicating that protein 4.2 deficient red cells were depleted specifically of band 3 molecules which were either unattached or bound with a low affinity to the membrane skeleton [15]. In addition, fluorescence recovery after use of the photobleaching (FRAP) method (see Section 5.1.2.5) has shown a shift in the lateral mobility of band 3, which is consistent with an increase in the mobile fraction of band 3 in membranes from individuals completely lacking protein 4.2, and with an increase in the mobile fraction of band 3 (Fig. 16.10). Total recovery (mobile fraction) dramatically increased up to almost 100% with nearly complete absence of the immobile component of band 3, as compared with normal subjects [15]. Another common feature of the FRAP curves in these patients was that a nearly linear slow recovery component has appeared in addition to the fast recovery component observed in normal red cell ghosts [15]. These changes indicate increased oligomerization of band 3, which has been suggested by increased larger IMPs.

EM studies were carried out to elucidate the abnormalities of red cell membrane ultrastructure *in situ* in total protein 4.2 deficiency [15] (see Section 16.2.2). When intact red cells were subjected to EM using the freeze fracture (FF) method (Fig. 16.11), the normal number of IMPs was $5\,210 \pm 389\ \mu\text{m}^{-2}$, of which approximately 80% were basically small in size (4–8 nm). On the other hand, in the red cells of protein 4.2 deficiency of the three types, the number of IMPs had decreased to $4\,464 \pm 53\ \mu\text{m}^{-2}$ in the Nippon type, $4\,625 \pm 381$ in protein 4.2 Shiga, and $2\,975 \pm 310$ in protein 4.2 Komatsu [11]. The decreased number of IMPs appeared to be derived from a decreased number of IMPs of small size in association with an increased number of IMPs of medium (9–20 nm) and large size (>21 nm), indicating increased oligomerization of band 3 *in situ* [11].

The significant contribution of protein 4.2 to the biophysical properties of band 3 was proved by utilizing inside-out vesicles (IOVs) of normal controls and those of protein 4.2 deficiency [11, 15]. In protein 4.2 deficiency, the distribution pattern of IMPs was totally deranged in IOVs. When spectrins and membrane proteins other than band 3 were stripped from the IOVs at pH 11 in the normal controls, this experimentally produced protein 4.2 deficiency demonstrated a markedly abnormal aggregation of band 3, which was the same as that in protein 4.2 deficient patients [11, 15].

The cytoskeletal network was also examined by EM using the quick-freeze deep-etching (QFDE) method [11, 15] (Fig. 16.12). The cytoskeletal network in the normal subjects showed numerous basic units, resembling “cages”, the number of which was $539 \pm 20\ \mu\text{m}^{-2}$. The “cage”-like structures consisted essentially of

two major types of units, i. e., small (20–44 nm), and medium (45–65 nm) sized units as determined by the interdistance (or diameter) of the longer axis of each structure. In the normal subjects, two-thirds of these units were of small size ($66 \pm 9\%$), and the remaining one-third were of medium size ($30 \pm 6\%$). There were only a few large-sized units ($4 \pm 1\%$) in the normal subjects. In contrast, in protein 4.2 deficiency, the uniform distribution of filamentous structures was lost, and apparent branchpoints of the filamentous elements were markedly distorted or disrupted. The number of cytoskeletal units was markedly reduced in protein 4.2 Komatsu ($195 \pm 38 \mu\text{m}^{-2}$), less in the Nippon type ($282 \pm 27 \mu\text{m}^{-2}$), and least in protein 4.2 Shiga ($339 \pm 35 \mu\text{m}^{-2}$) [11, 15]. In protein 4.2 deficiencies, the cytoskeletal units of the basic small size (20–44 nm) were markedly reduced in protein 4.2 Komatsu ($25 \pm 4\%$), in the Nippon type ($27 \pm 5\%$), and in protein 4.2 Shiga ($34 \pm 5\%$), compared with the normal controls ($66 \pm 9\%$). In their place, units of large size (69–92 nm) and of extra-large size (93–180 nm), which were essentially not present in the normal subjects, were increased tremendously in these protein 4.2 deficiencies.

Under normal conditions, the cytoskeletal network is believed to be stabilized by tightly binding to band 3 molecules via ankyrin [15] (see Sections 5.1.2.5 and 6.1.3). Two-thirds of band 3 is immobilized by this binding, the remaining one-third is mobile and unfixed without binding to the cytoskeletal network. In the absence of protein 4.2, the cytoskeletal network appeared to become extremely unstable due to the loss of integrity of its small basic units, resulting in disruption of the interconnected structure of the cytoskeletal network. Under this pathological condition with a markedly impaired cytoskeletal network, band 3, two-thirds of which is normally connected with the cytoskeletal network mainly via ankyrin, should lose its binding to the network and become unfixed and mobile. It is known that free band 3 molecules tend to aggregate or cluster. The increased large sizes of the IMPs in protein 4.2 deficiency may be the result of aggregation and/or clustering of these increased mobile band 3 molecules, which were initially immobile band 3 bound to the cytoskeletal network [11, 15]. The aggregated or clustered band 3 should naturally produce a decrease in the apparent number of IMPs.

The red cell membrane protein 4.2 gene was targeted in embryonic stem (ES) cells to create a null mutation ($4.2^{-/-}$) in mice (see Section 15.1.3). A fragment extending from intron 3 to exon 8 was replaced with a neomycin-resistant cassette, removing exons 4 through 7 and part of exon 8. Homozygous null mutations were not distinguishable from normal littermates by phenotype at any age. Protein 4.2 was not detected in $4.2^{-/-}$ red cell ghosts [76]. No protein 4.2 mRNA was detected in $4.2^{-/-}$ newborn reticulocyte RNA [76]. Hematologically, $4.2^{-/-}$ mice had mild HS with increased reticulocytosis ($5.5 \pm 0.8\%$: control $2.5 \pm 0.1\%$). Red cell morphology demonstrated the presence of spherocytosis in $4.2^{-/-}$ mice [76]. In protein chemistry, the band 3 content of $4.2^{-/-}$ red cells appeared to be decreased, but normal amounts of spectrin, ankyrin, protein 4.1, p55, and glycophorin C were observed. Ultrastructural findings in these protein 4.2 $^{-/-}$ mice confirmed almost the same observations as observed in human protein 4.2 deficiency.

10.4.4

Isolated Partial Spectrin Deficiency

As described previously, spectrin is composed of two subunits, the α -chain and β -chain, which are structurally distinct and are encoded by separate genes, despite the many similarities (see Section 4.1). Red cell spectrin demonstrates several cellular functions, i. e., (1) as a determinant of cell shape, (2) as a regulator of the lateral mobility of integral membrane proteins, and (3) as a supporter for the lipid bilayer in the three dimensional membrane ultrastructure (see Section 3.2.2).

Synthesis of α -spectrin in humans is about three times more excessive than that of β -spectrin (see Chapter 7). Therefore, β -spectrin is rate-limiting in the overall spectrin synthesis. If patients who are heterozygous for an α -spectrin defect, they are still able to produce enough normal α -spectrin chains to pair with all of the β -spectrin chains synthesized. Thus, one can expect that patients with α -spectrin defects should only be symptomatic when the defect exists in the homozygous or compound heterozygous state. Along the same line of discussion, deficiency of the limiting β -spectrin chains due to β -spectrin defects should be expressed as a dominantly inherited trait.

Spectrin deficiency in HS patients was observed in both the dominant [77–85] and also recessive [10] inheritance forms (Table 10.1). In general, the degree of spectrin deficiency correlates with the extent of microspherocytosis, the ability to resist against shear stress, the degree of hemolysis, and the response to splenectomy.

Regarding the α -spectrin anomaly in the HS patients, α -spectrin mutations [88–92] appear to be rare. However, a patient with severe HS has been reported, who was a compound heterozygote for two different α -spectrin gene mutations [86–90]. In one allele, there was a mutation of α -spectrin Prague [89], and in the other allele there was another mutation of α -spectrin LEPRa (*Low Expression PRa-gue*) [90]. This variant, a C→T substitution 99 nucleotides upstream of the acceptor splice site of intron 30 of the α -spectrin gene, produces an aberrantly spliced α -spectrin mRNA. The variant allele produces approximately six-fold less of the correctly spliced α -spectrin transcript than the normal allele. In the heterozygous state, α -spectrin LEPRa is asymptomatic, as expected. The combination of α -spectrin LEPRa with other mutations of α -spectrin *in trans*, leads to significant α -spectrin deficiency and severe anemia with microspherocytosis. In many patients with nondominant, spectrin-deficiency in HS patients, α -spectrin LEPRa is in linkage disequilibrium with α -spectrin Bughill [87, 88], which demonstrates an amino acid substitution in the α II domain of the α -spectrin gene, and is not itself responsible for HS. The two other reports on the same line have recently been published [91, 92] implicating that the combination of α -spectrin LEPRa and α -spectrin allele encoding a nonfunctional peptide may produce severe nondominant HS.

A lethal and near lethal HS which was associated with serious (26 % of normal) spectrin deficiency showed a markedly decreased α -spectrin synthesis at the stage of burst-forming unit-erythroid (BFU-E) during erythroid differentiation. The proband appears to possess at least two genetic defects that in a simple heterozygote have essentially no adverse consequences.

Mutations of the β -spectrin gene [10, 78–82] have been identified in a number of patients with dominantly inherited HS associated with spectrin deficiency (Table 10.1). These mutations are basically private and unique to individual kindred, except for β -spectrin Houston [10], which has been found in patients from several unrelated kindred. Therefore, β -spectrin Houston is a frameshift mutation due to a single nucleotide deletion of the β -spectrin gene, and might be a common β -spectrin mutation associated with HS.

Amongst the various types of gene mutations, truncated β -spectrin chains due to genomic deletions, exon skipping, and frameshift mutations have now been identified. A few missense mutations associated with HS have been described. Spectrin Kissimmee [84, 85] is one of these missense mutations, and is an unstable β -spectrin that cannot bind protein 4.1 and binds poorly to actin due to a point mutation in a highly conserved region of β -spectrin probably at the region of protein 4.1 binding.

In some HS patients with the β -spectrin gene mutations, the presence of a subpopulation of acanthocytes [10] as well as microspherocytes is noted.

10.5

Cellular Phenotypes: Spherocytosis and Membrane Transport

Spherocytosis is a true indicator of HS. Typical spherocytes, especially microspherocytes, demonstrate a decreased surface-to-volume ratio, which comes from loss of membrane surface. HS red cells are caused by local disconnection of the skeleton and lipid bilayer, which is followed by membrane vesiculation of the surface components at the unsupported membrane regions. Thereafter, progressive reduction in membrane surface area is accelerated leading to the formation of spherocytes. In fact, the lipid contents of the phospholipids and free cholesterol in HS red cells of the unsplenectomized patients are significantly decreased; i.e., $2306 \pm 169 \mu\text{g}$ per 10^{10} red cells in total phospholipids (normal: 2600 ± 220), and $1084 \pm 63 \mu\text{g}$ per 10^{10} red cells in free cholesterol (normal: 1203 ± 93) [93, 94]. These values of isolated spherocytes are more markedly reduced and it clearly indicates the loss of surface area in these HS red cells. HS red cells lose their membrane more readily than normal when metabolically deprived or when their ghosts are subjected to conditions facilitating vesiculation.

It is now known that the molecular basis of HS is heterogeneous, and that surface area deficiency is a consequence of several distinct molecular mechanisms resulting in weakened vertical connections between the skeleton and the lipid bilayer membrane. Two principal hypotheses have been proposed, based on the biochemical results on the abnormalities in HS membranes [95]. In the first hypothesis, the lipid bilayer and integral membrane proteins are directly stabilized by their interaction with ankyrin or the spectrin skeleton. In patients with isolated spectrin deficiency or a combined deficiency of ankyrin and spectrin, the surface area deficiency appears to be associated with an uncoupling of the lipid bilayer membrane from the underlying skeleton. Spectrin deficiency, directly or indirectly due to ankyrin deficiency, appears to lead to a decreased density of the skeletal network (Fig.

16.3) which lies underneath the lipid bilayer membrane. Consequently, unsupported areas of the lipid bilayer membrane by the skeletal network are susceptible to release from the cells as microvesicles. Cell surface area and membrane stability are proportional to red cell spectrin content and are decreased in HS red cells. Therefore, spectrin-deficient spherocytes are believed to be less resistant against shear stress in the peripheral circulation and to be subjected to splenic sequestration.

In the second hypothesis regarding the role of band 3, membrane surface area is stabilized by interactions of band 3 with neighboring lipids. Band 3 protein is known to span many folds of the lipid bilayer membrane. Therefore, in the band 3 deficient state, a substantial amount of boundary lipid is released together with the band 3 protein molecules, which leads to a loss of surface area. It is also possible that, in band 3 deficiency, band 3-free regions can be produced in the membrane, which are followed by the formation of membrane blebs. The membrane will be subsequently released from the cells as microvesicles. This is typically observed in red cells of bovine band 3 deficiency [58] (Figs. 15.1 and 15.5) and also of targeted band 3 knockout mice [59, 64]. Red cells totally lacking band 3 molecules released membrane vesicles by marked microvesiculation, probably due to the distinct instability of the membrane ultrastructure.

It is believed that HS red cells are intrinsically more leaky to Na^+ and K^+ ions than normal red cells [96]. A similar abnormality is observed in spectrin-deficient mice [97]. In fact, the excessive Na^+ influx, increased activity of Na^+ , K^+ -ATPase, and the accelerated ATP turnover and glycolysis are known in HS red cells. However, the extent of the Na^+ flux does not correlate with the extent of hemolysis in HS patients [98]. Furthermore, patients with hereditary hydrocytosis with a much more enhanced Na^+ permeability do not develop microspherocytosis, but rather develop marked cell hydration. In contrast, HS red cells are significantly dehydrated cells even with increased Na^+ permeability. Therefore, this modest Na^+ leak *per se* is not responsible for the increased hemolysis of HS red cells although the pathways causing HS red cell dehydration have not been clearly defined.

10.6

Role of the Spleen

The presence of splenomegaly had already been recognized in 1871 by Vanlair and Masius (see Section 1.2). The pathogenesis is composed of two factors: (1) membrane abnormalities of HS red cells, and (2) the contribution of the spleen.

The impaired red cell deformability is derived from microspherocytosis with a decreased cell surface to cell volume ratio. HS red cells lack cell surface area sufficient for passing through narrow microcirculation openings in the spleen, which is available in normal red cells. The HS red cells are entrapped at fenestrations in the wall of splenic sinuses, where blood from the splenic cords of the red pulp enters the venous circulation [4–6, 95, 99]. The length and width of these fenestrations are 2–3 μm and 0.2–0.5 μm , respectively. The size of these openings is about

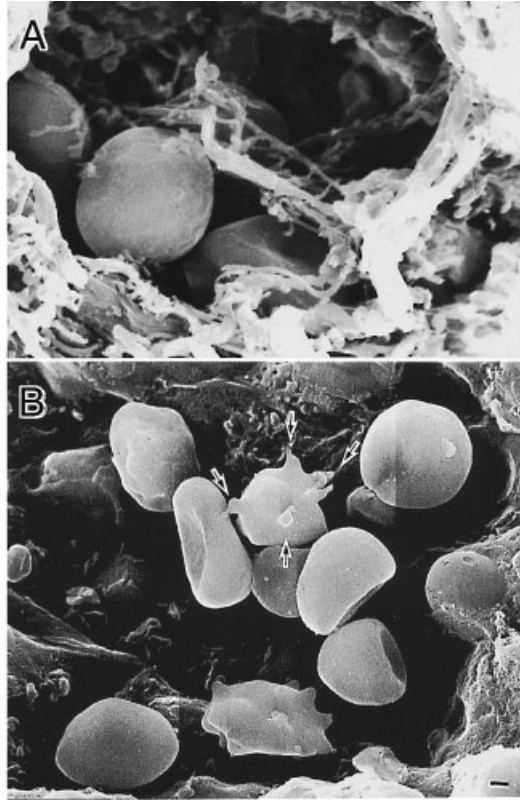


Figure 10.2 Sequestration of spheroid cells in the splenic sinus in patients with hereditary spherocytosis. A and B are scanning electron micrographs in the spleen taken from patients with hereditary spherocytosis. A marked exocytosis is observed in spheroid red cells as indicated by arrows.

one-half the red cell diameter. The less deformable spheroid cells are entrapped in the red pulp of the specimens of the spleen which were obtained from an HS patient after splenectomy, as shown by the electron micrographs (Fig. 10.2).

The relative percentage of various red cell shapes in peripheral blood of HS patients is $38.0 \pm 5.2\%$ in discocytes, $31.3 \pm 3.9\%$ in disco-stomatocytes, $13.5 \pm 1.8\%$ in stomatocytes, $5.4 \pm 1.8\%$ in stomato-spherocytes, $10.3 \pm 0.7\%$ in spherocytes, and $1.5 \pm 0.3\%$ in poikilocytes, respectively. In red cells in the splenic vein, discocytes are 33.7% , disco-stomatocytes 18.8% , stomatocytes 17.8% , stomato-spherocytes 13.4% , spherocytes 14.5% , and poikilocytes 1.8% , respectively. In the intrasplenic blood, discocytes are $18.4 \pm 2.2\%$, disco-stomatocytes $21.3 \pm 1.2\%$, stomatocytes $22.1 \pm 2.6\%$, stomato-spherocytes $14.6 \pm 1.5\%$, spherocytes $18.7 \pm 1.1\%$, and poikilocytes $4.9 \pm 0.5\%$, respectively. These results clearly indicate the marked accumulation of spherocytes concomitant to the significant decrease of discocytes inside the spleen. Even in the splenic vein, there is the same tendency as that in the spleen but to a lesser extent.

After the entrapment of spherocytes inside the spleen, the HS red cells are subjected to metabolic stress with low pH, decreased glucose concentration, increased

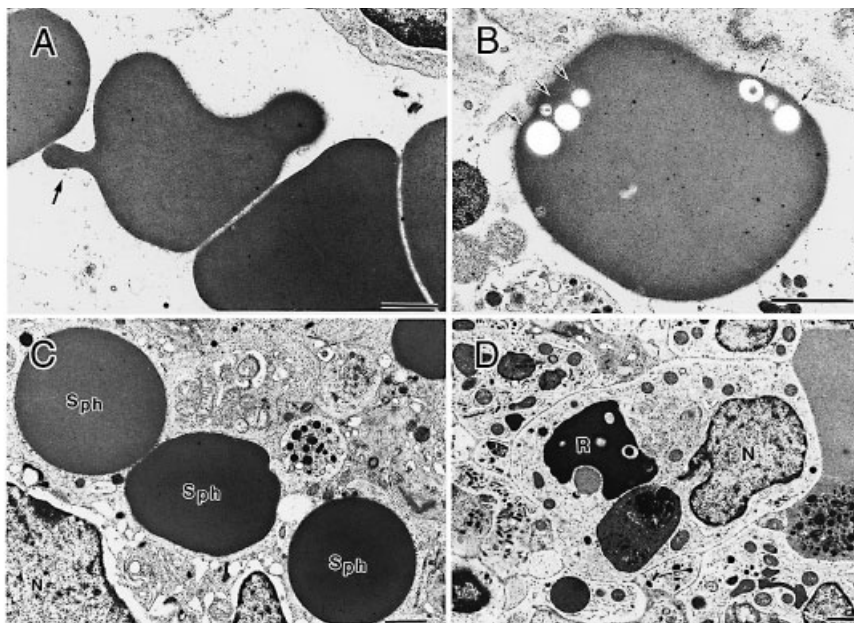


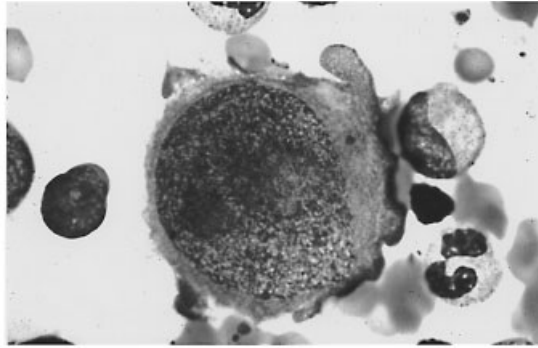
Figure 10.3 Electron micrographs of the splenic specimens examined by transmission electron microscopy. A: exocytosis in a red cell is shown by an arrow. B: marked endocytosis in a spherocyte is indicated by arrows. C: Three phagocytized spherocytes (Sph) by a macrophage in the spleen. D: Red cells (R), which are phagocytized, are partially digested in the cytoplasm of macrophage. N: nucleus.

lactate level, and low oxygen tension in or near the splenic sinuses [4–6, 95]. In these circumstances, HS red cells undergo additional damage marked by further loss of surface area and an increased cell density (dehydrocytes). The other important factor is the presence of macrophages lined up on the vascular walls of the spleen. Electron microscopy demonstrates clearly the presence of the phagocytic process of spherocytes by macrophages (Fig. 10.3). The detailed mechanism of this phagocytosis with the HS red cell surface alterations are still unclear. Altered phospholipid asymmetry may take place in the end-stage HS red cells, which are repeatedly conditioned in the spleen.

10.7 Complications

Gallstones as a result of hyperbilirubinemia are found in 22.2 % of Japanese HS patients, and often in up to 50 % of patients, even in those with a very mild form of the disorder [7]. The bilirubin stones are usually uncommon in very young children [4–6, 95]. Since the incidence of this complication is high, HS patients should be periodically examined by ultrasound equipment for the presence

Figure 10.4 A giant proerythroblast in the bone marrow was present in a patient with parvovirus B19 infection (originally stained by the Wright–Giemsa method).



of gallstones. When a splenectomy is scheduled, the concomitant surgical operation of cholecystectomy will be recommended [100].

During its clinical course, a sudden episode of severe anemia may occur as a complication, mostly resulting from pure red cell aplasia by parvovirus B19 infection (aplastic crisis) or from an acute hemolytic crisis [101]. The parvovirus infection is also called an erythema infectiosum, or fifth disease, the clinical features of which are fever, chills, lethargy, malaise, nausea, vomiting, abdominal pain with occasional diarrhea, respiratory symptoms, muscle and joint pains, and a maculopapular rash on the face, trunk and extremities [4–6, 95]. The parvovirus B19 virtually selectively infects erythroid precursors and inhibits their proliferation and differentiation, and the ensuing anemia. Erythroid replication is arrested in the S phase of the cell cycle by selective cytotoxic invasion of the virus into erythroblasts, resulting in a severe, life-threatening acute aplastic crisis [101]. Since this is an endemic infection, multiple family members of the HS kindred can be infected and develop aplastic crises at the same time. This parvovirus infection can also be associated with neutropenia and thrombocytopenia. Infection with this virus is dangerous particularly to susceptible pregnant women because it can infect the intrauterine fetus, ensuing serious fetal anemia, which may lead to hydrops fetalis, and fetal miscarriage [4–6, 95]. Aplastic crisis was observed in 2% of Japanese HS patients. When giant proerythroblasts present in the patient's bone marrow (Fig. 10.4) are examined by transmission electron microscopy, virus particles in the nucleus are clearly observed. Through genomic analyses of the virus, its amino acid sequence has been established. A nonstructural protein (77 kDa) of the virus appears to be responsible for the disturbance of erythropoiesis.

Hemolytic crisis occurs fairly often, and was observed in 25.7% of HS patients [7] in association with fatigue, flu-like infection, pregnancy, parturition, the administration of some drugs, or unknown etiology [4–6, 95].

Hematologic malignancies, such as myeloproliferative disorders, leukemias, and myelodysplastic syndrome, have been reported in HS patients [4–6, 95]. The pathogenesis that is speculated is that long-standing hematopoietic stress is predisposed to the development of these secondary complications, although it is uncertain if these complications occurred at random.

As described previously, even in the HS patients with the more severe form, leg ulcers and extramedullary masses were not present in our nationwide survey in Japanese HS patients [7]. Thus, these complications may exist, but appear to be extremely rare, compared with those in severe hemoglobinopathies.

10.8

Therapy and Prognosis

The removal of the spleen, which is the major site of the destruction of the HS red cells, is undoubtedly the first choice of treatment for HS [4–7, 100].

In the nationwide survey for HS by the Committee for Studies on Hemolytic Anemias assigned to the Japanese Ministry of Health and Welfare (1974–1977), 60.2% of the HS patients was subjected to splenectomy [7]. The final outcome showed that nearly complete remission was obtained in 68.8% of these HS patients, effective results in 16.9%, and minimal effects in 3.9%, respectively [7]. Thus, beneficial results through splenectomy were obtained in 89.6% of the HS patients. These patients were splenectomized within 2 years of the diagnosis of HS being established.

Splenectomy is the best treatment for HS [5, 100]. After splenectomy, red cell counts were improved to $4.44 \pm 0.64 \times 10^6 \mu\text{L}^{-1}$ from $3.07 \pm 0.62 \times 10^6 \mu\text{L}^{-1}$ before splenectomy, Hb to $13.7 \pm 1.7 \text{ g dL}^{-1}$ from $9.9 \pm 2.1 \text{ g dL}^{-1}$, Hct to $39.5 \pm 4.4\%$ from $27.8 \pm 5.4\%$, reticulocytes $2.1 \pm 4.5\%$ (or $0.09 \pm 0.17 \times 10^6 \mu\text{L}^{-1}$) from $12.2 \pm 10.0\%$ (or $0.37 \pm 0.32 \times 10^6 \mu\text{L}^{-1}$), total bilirubin $0.90 \pm 0.50 \text{ mg dL}^{-1}$ from $4.00 \pm 2.12 \text{ mg dL}^{-1}$, and apparent half-life of red cell survival ($T_{1/2}$) to 22.4 ± 6.2 days from 9.9 ± 4.0 days, respectively [7]. The spleen size was $606 \pm 378 \text{ g}$ in these HS patients [7].

Based on these results as described above, splenectomy is significantly effective in patients with typical forms of HS. However, red cell membrane abnormalities *per se* persist, such as microspherocytosis and the increased osmotic fragility [4–7, 96].

At the time of splenectomy, it is also preferable to carry out a cholecystectomy, especially in patients with frequent gallstone episodes [100].

The benefits and risks of splenectomy must be considered carefully in HS patients [5, 100]. Generally speaking, the morbidity of splenectomy as a surgical practice is clearly lower in HS than in other hematological disorders. The benefits of surgery, however, should be evaluated carefully against possible complications, especially postsplenectomy infections, including an increased frequency of penicillin-resistant pneumococci infection and the postsplenectomy sepsis syndrome [4–6, 95]. These complications are actually rare, and their frequency can also be reduced by appropriate vaccinations against *Pneumococcus* (Pnu-Imune 23 or equivalent, 0.5 mL intramuscularly or subcutaneously) with polyvalent vaccine as well as with vaccines against *Hemophilus influenzae* type b and *Meningococcus* (Menomune-A/C/Y/W–135, 0.5 mL intramuscularly) [100]. After splenectomy, prophylactic antibiotics (penicillin V 250 mg orally twice a day for adult patients) are

recommended [101]. HS patients should be given folic acid (1 mg a day orally) to prevent folate deficiency due to increased erythropoiesis [100]. Early complications of splenectomy should not be ignored, such as local infection, bleeding, and pancreatitis. Therefore, splenectomy may not be recommended in HS patients with mild to moderate severity, who are younger than 3–5 years of age, because of an as yet incomplete immune system [4, 5, 100].

After splenectomy, clinical features of HS may reappear due to a relapse of this disorder by the enlargement of an accessory spleen, which escaped from the surgical resection. In some occasions, the reevaluation of diagnosis of HS *per se* may be required, when splenectomy was not effective [4–7, 100].

Laparoscopic splenectomy has been recommended by several surgical specialists, mainly from the cosmetic stand point [5, 100]. The disadvantages, however, should also be seriously considered: exposure of the patient to many hours under anesthesia for the operation, especially in cases with marked splenomegaly; the possibility of overlooking an accessory spleen, which would induce a relapse; and difficulty in controlling a tendency to hemorrhaging [5, 100].

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11

Hereditary Elliptocytosis (HE)

11.1

Definition and Epidemiology

Hereditary elliptocytosis (HE) is a group of inherited disorders that demonstrate the characteristic feature of the presence of elliptocytes on blood films obtained from peripheral blood [1–4]. The red cell morphology varies from so-called “rod-shaped” red cells to oval-shaped red cells (ovalocytes) (Fig. 11.1). This disorder appears to be a relatively common, clinically heterogeneous disorder. In the more severe form of this disorder, spherocytes or poikilocytic bizarre-shaped red cells are also present, especially in hereditary pyropoikilocytosis (HPP) [1–4].

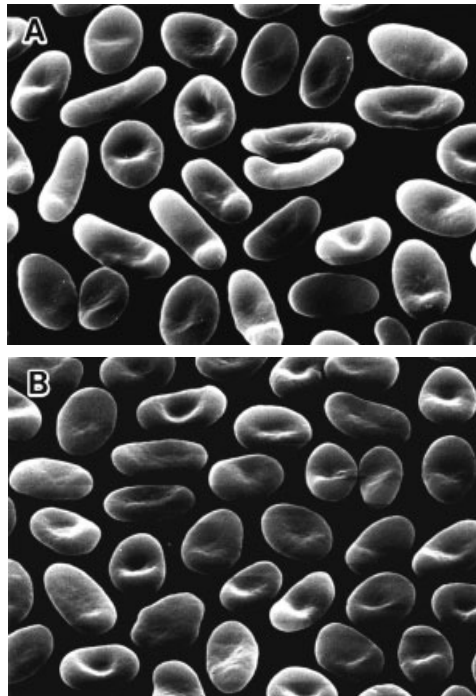


Figure 11.1 Scanning electron micrographs of hereditary elliptocytosis. A, rod-shaped type, B, ovalocytic type.

It is known that elliptocytosis was first reported by Dresbach in 1904 [5] followed by Hunter and Adams who established the hereditary property of this disorder in 1929 [6].

It should be mentioned that elliptocytosis *per se* is not necessarily associated with a hemolytic nature [1–4]. Substantial numbers of individuals with hereditary elliptocytosis do not demonstrate any significant hemolytic anemia. Therefore, considerable debate has taken place regarding the question of whether this disorder of elliptocytosis is a disease or a simple morphological polymorphism [1–4]. In fact, in nature, elliptocytosis itself has widely been recognized in reptiles, birds, camels and many others, where it is not associated with any hemolytic anemia, nor with hemolytic episodes [7]. In reptiles and birds, elliptocytic cells in peripheral circulation are composed of nucleated red cells [7].

The true incidence of HE is not really known because its clinical severity varies, and many individuals with typical elliptocytosis are asymptomatic and do not show hemolytic anemia. The prevalence of HE in the population in the USA is estimated to be about 3–5 per 10 000 [1–4]. Based on the results from the survey study by the Hemolytic Anemia Study Committee assigned to the Japanese Ministry of Health and Welfare in 1974 [8], the relative incidence of hemolytic HE compared with that of HS was seven cases to 181 cases of HS, although no field study was performed. At my own laboratory at the Kawasaki Medical School, the number of kindred and patients with HE was 68 (11.2%) and 137 (13.5%) out of 605 kindred and 1014 patients with red cell membrane disorders of hereditary origins (in 1975–2000), respectively [9]. The relative incidence of HE compared with HS was 1:4.5 in the kindred and 1:4.2 in the patients among these two disorders.

Hereditary elliptocytosis is generally an autosomal dominantly inherited disorder [1–4]. Homozygous or compound heterozygous patients for HE usually demonstrated a more severe phenotype with increased hemolysis and splenomegaly. Heterozygous patients appear to be mild or even asymptomatic in their clinical phenotype.

Hereditary pyropoikilocytosis (HPP), which was first described [10] in children with congenital hemolytic anemia with fragmented and irregularly shaped red cells when heated, is now thought to be a severe form of HE. HPP is a much rarer disorder. The patients are expected to be homozygotes for certain HE genes, compound heterozygotes for two different HE alleles, or coinheritants of a certain HE gene and a low expression gene as a gene modifier, such as the α^{LELY} allele (Low Expression gene LYon) (see Sections 4.1, 11.4 and 14.1.2).

Another interesting disorder of this category is Southeast Asian ovalocytosis (SAO), which is also a distinct and homogeneous subgroup of HE prevailing endemically in some parts of Southeast Asia, especially in Malaysia and Polynesia [11] (see Section 11.5 in detail).

An elliptocytosis locus (EL1) has been considered to be closely linked to the Rh locus on chromosome 1p34–p36 (Table 5.1), which is now known to be the gene for protein 4.1 (1p36.1) (Table 1.2). Another locus (EL2) has been reported on chromosome 1 near the Duffy blood group antigen locus (1q22–q23) (Table 5.1), which is now known to be assigned to the α -spectrin gene (1q22–q23) (Table 1.2). The

third locus has recently been suggested to be an X-linked elliptocytosis locus (band q22 in the X chromosome), which is associated with Alport syndrome [12].

11.2

Clinical and Laboratory Findings

The HE syndrome is divided into several subgroups based on clinical severity and other characteristic features [1–4]. Molecular and genetic abnormalities in different membrane proteins usually induce a similar clinical phenotype, probably disrupting red cell membrane integrity, in particular red cell deformability, through a uniform mechanism.

The most common type of HE is known as common HE, typically with autosomal dominant inheritance [1–4]. The clinical phenotype is usually mild with neither anemia nor splenomegaly. Red cell survival is basically not shortened. In some patients, there may be very mild, compensated hemolysis, a slight reticulocytosis, and a slightly increased indirect bilirubin with a decreased haptoglobin. A striking feature of this common HS is marked elliptocytosis (usually 50–90%) without poikilocytosis, budding, fragmentation, or spherocytosis (Fig. 11.1). The extent of elliptocytic transformation is typically striking, such as very elongated elliptocytes (rod-shaped red cells), which are present at from 2 to 5% in normal peripheral blood. The rod-shaped elliptocytes are present mostly in the HE patients without hemolysis ($21.1 \pm 11.4\%$ of total elliptocytes) rather than in those with overt hemolysis ($2.1 \pm 1.7\%$) [13]. Instead, hemolytic HE tends to demonstrate ovalocytosis rather than rod-shaped red cells in peripheral blood. Stomatocytic changes, which are superimposed on elliptocytosis, are less striking in non-hemolytic HE ($21.2 \pm 7.9\%$ of the total red cells) than in hemolytic HE ($30.4 \pm 13.9\%$) [13]. The clinical phenotype in common HE without overt hemolysis demonstrates $4.20 \pm 0.70 \times 10^6 \mu\text{L}^{-1}$ of the red cell count, $1.8 \pm 0.9\%$ of reticulocytes, $34.2 \pm 0.7\%$ of the mean corpuscular hemoglobin concentration (MCHC), and $0.3 \pm 0.1 \text{ mg dL}^{-1}$ of the indirect bilirubin level, compared with those in hemolytic HE, such as $2.87 \pm 0.69 \times 10^6 \mu\text{L}^{-1}$, $5.5 \pm 4.6\%$, $35.0 \pm 2.2\%$, and $3.7 \pm 3.1 \text{ mg dL}^{-1}$, respectively [13].

It is known that elliptocytic transformation in common HE may become prominent with time, is less frequent in the cord blood of infants, and much more in adults [1–4].

Although typical common HE does not usually demonstrate any clinical symptoms, a more severe, uncompensated anemia may develop in a minority of the patients with common HE. Acute hemolytic episodes may be evoked by infections (viral or bacterial), pregnancy and delivery, malaria, cirrhosis, and many other pathogeneses [1–4].

Chronic uncompensated hemolysis may also be observed in common HE. The pathogenesis is still unclear in most cases, except for a genetic modifying factor such as the low-expression gene α^{LEY} (Low Expression gene LYon) (see Sections 4.1, 11.3.3 and 14.1.2).

Some infants with common HE suffer from moderately severe hemolytic anemia with marked red cell poikilocytosis and neonatal jaundice. Elliptocytosis is usually less striking. It has been reported that elevated free 2,3-diphosphoglycerate (2,3-DPG) in fetal red cells increases the fragility of isolated red cell ghosts, and weakens actin–protein 4.1 binding, protein 4.1–glycophorin C, and ankyrin–band 3 binding [14]. Therefore, this mechanism may play some role in disturbing the formation of the normal cytoskeletal network, especially spectrin self-association.

The laboratory findings on common HE is not remarkable, except for prominent elliptocytosis, most of which are rod-shaped red cells. Conventional studies in clinical hematology reveal no substantial abnormalities. Osmotic fragility is usually normal, and ektacytometry yields normal red cell deformability in common HE. Thermal hypersensitivity of red cells is not observed.

The next clinical phenotype is severe HE, which resembles hereditary pyropoikilocytosis (HPP). The patients are homozygous or compound heterozygous HE, and demonstrate a very severe or even fatal transfusion-dependent hemolytic anemia with marked red cell fragmentation, poikilocytosis, spherocytosis, and elliptocytosis [1–4].

HPP is characterized by a severe hemolytic anemia in infancy or early childhood with extreme poikilocytosis, striking bud formation and fragmentation [10, 15, 16]. Spherocytes, elliptocytes, triangular red cells and other bizarre-shaped red cells are seen in the peripheral circulation. When this severe anemia persists for many years especially in infancy and childhood, long-term complications of severe anemia will be growth retardation, frontal bossing, and cholelithiasis in the early stage of this HPP. The most characteristic feature of HPP is the prominent thermal sensitivity. Normal red cells do not fragment until 49 °C when they are subjected to heat treatment for a short period of time [10]. Normal red cells become unstable and fragment spontaneously at the temperatures above 50 °C probably because of denaturation of spectrin. HPP red cells start to fragment after 10 min at 44–46 °C. These red cells fragment even at 37 °C under prolonged heat treatment for 6 h. Purified spectrin from HPP red cells is heat-sensitive, implying that an increased sensitivity of spectrin to heat denaturation appears to be pathognomonic for HPP. It has also been reported that the red cells of severe HE or HPP are deficient in spectrin, by up to 30 % less than the normal level [17]. Osmotic fragility is definitely abnormal in severe forms of HE and HPP reflecting a loss of red cell membranes due to their fragmentation. Ektacytometry demonstrates markedly decreased red cell deformability. In severely affected patients with HPP, the MCV is very low (indicating severe microcytosis) because of the presence of numerous fragmented red cells.

The third clinical phenotype of HE is spherocytic HE [1–4]. This subgroup appears to be a hybrid of HE and HS. This condition is reasonably rare, and the extent of hemolytic anemia is mild to moderate. The red cell morphology indicates a mixture of some spherocytes, microspherocytes, and microelliptocytes. The clinical expression of this spherocytic HE is similar to that of classical HS, showing increased osmotic fragility and increased autohemolysis, which is protected in the presence of glucose. Splenic sequestration of the abnormal red cells is definitely present just as in HS.

The fourth clinical phenotype of HE is a distinct entity, Southeast Asian ovalocytosis (SAO) [11, 18, 19]. This disorder is found mainly in the aboriginal populations of Melanesia, Papua New Guinea, and other related regions. The heterozygous individuals are usually asymptomatic or have mild compensated hemolysis. Homozygosity appears to be lethal [20]. A red cell morphology is unique; that is, ovalocytes (round elliptocytes) of 20–50% of the total peripheral red cells, some of which have one or more traverse bars that divide the central portion of biconcave ovalocytes. SAO red cells are rigid rather than deformable due to increased rigidity and decreased deformability of the red cell membrane [21]. SAO red cells are also heat-stable, and osmotically resistant [22]. It is interesting to note that many blood group antigens (I^T , I^F , LW, D, C, e, S, s, U, Kp^b , Jk^a , Jk^b , Xg^a , Wr^b , Scl, En^a , etc.) are deficient or poorly expressed on the surface of SAO red cells [23]. There is a decreasing SAO prevalence in malaria patients with the more severe disease, suggesting that the rigid SAO red cells are resistant to invasion by malaria parasites [22].

X-linked elliptocytosis has recently been reported in four members of an English family with a deletion of band q22 in the X chromosome [12]. These members have Alport syndrome due to deletion of the COL4A5 gene. Mental retardation, dysmorphic faces with midface hypoplasia, and marked elliptocytosis were observed in the two affected male members. However, there was no anemia with normal reticulocytes and normal red cell membrane fragility. The detailed pathogenesis remains to be elucidated in the near future.

11.3

Pathogenesis: Affected Proteins and Their Related Gene Mutations

11.3.1

Overall Pathogenesis

Although much extensive clinical and scientific information has been accumulated, the exact pathobiology of the elliptocytic shape is still not really understood in detail. In hereditary elliptocytosis, nucleated erythroid precursors are round in shape, and elongate or fragment during peripheral circulation of their red cells, when burst-forming unit erythroids (BFU-E), which are obtained from the peripheral blood of HE patients, are incubated by the two-phase liquid culture method [24]; newly-formed erythroblasts and red cells are round and discoid in shape. This process resembles the gradual development of spherocytosis in hereditary spherocytosis. These observations support the hypothesis that the process of the morphological change is secondary to the intrinsic structural instability of the skeleton. Skeletal defects appear to lead to elliptocytosis by increasing membrane plasticity. Normal red cells are able to recover their discoid shape reversibly, even after they are subjected to transient deformation forces. Spectrin–spectrin or spectrin–actin interactions realign, responding to prolonged shear stress. However, the HE red cells with defective horizontal skeletal interactions demonstrate an irreversible change in shape of the membrane skeleton when under the same con-

dition as normal red cells. The red cells with more severe horizontal skeletal defects such as HE or HPP red cells tend to become elliptocytic, and unable to withstand the shear stresses present during normal circulation. Even though the exact molecular mechanisms are not well defined in the different subtypes of HE, the processes that produce a shortened red cell life span should be identical to those in the various hereditary hemolytic disorders. Splenectomy is unequivocally effective, although the molecular mechanisms for splenic sequestration, “splenic conditioning”, and red cell destruction have not been well defined.

The principal defect in HE and HPP red cells is mechanical weakness or increased fragility of the red cell membrane skeleton. In HE, numerous abnormalities of various red cell membrane proteins have been identified [1–3]. The major membrane proteins pathognomonic for this disorder are α -spectrin, β -spectrin, protein 4.1, and glycophorin C (GPC). The majority of molecular defects are found in spectrins, which are the principal structural proteins of the red cell membrane skeleton. Most spectrin defects in HE and HPP are the impairment of the ability of spectrin dimers to self-associate into tetramers and oligomers, leading to disruption of the membrane skeleton. Abnormalities of protein 4.1 primarily induce disruption of the spectrin–actin attachment to the membrane through glycophorin C. The mechanical instability in abnormal glycophorin C, therefore, appears to be due to a secondary deficiency of protein 4.1. Whichever of these membrane proteins are affected, the membrane skeleton is disrupted, resulting in mechanical instability which causes red cell fragmentation with increased hemolysis under conditions of normal shear stress in peripheral circulation.

11.3.2

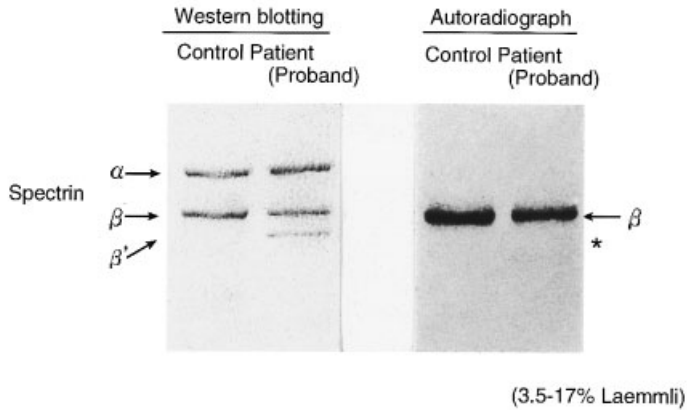
Analysis of Membrane Protein Abnormalities

To detect molecular abnormalities of red cell membrane proteins in HE, the primary method is electrophoretic separation of solubilized membrane proteins (see Section 2.3.1 and Fig. 1.3). In red cell membrane disorders, sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS–PAGE) is the first step towards detecting abnormalities of membrane proteins through the abnormal electrophoretic mobility of these proteins, which is identified by Western blotting with antibodies specific for these membrane proteins (Fig. 11.2). This procedure aims to detect truncated α - or β -spectrins in HE and HPP, truncated or elongated forms of protein 4.1, and a partial or complete deficiency of protein 4.1 in HE. A partial deficiency of spectrin can be detected by a decreased spectrin/band 3 ratio or a decreased spectrin content on each red cell from the results with SDS–PAGE gels, especially in HPP. Glycophorin C, one of the glycoproteins, can be detected on the SDS–PAGE gels with periodic acid–Schiff (PAS) staining, through which a deficiency of glycophorin C can be detected in a rare individual with a mild recessively inherited HE. Glycophorin C deficiency is also observed in protein 4.1-deficient HE patients.

To identify the most common functional abnormality in HE, analysis of the ratio of tetrameric spectrin (SpT) and dimeric spectrin (SpD) in low ionic strength extracts is usually carried out (Fig. 11.3). The procedure aims to detect weakened

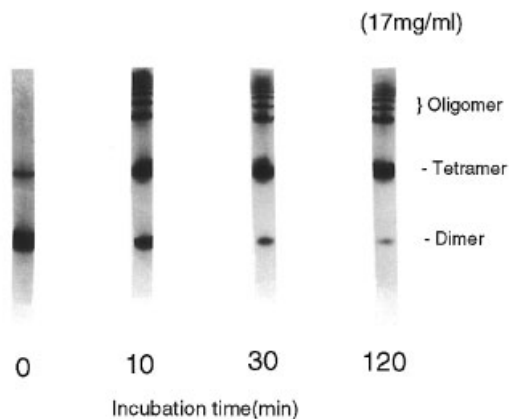
Figure 11.2

Detection of mutated β -spectrin by Western blotting with anti-spectrin antibody, and its state of phosphorylation in β -spectrin Le Puy in Yamagata. α : α -spectrin, β : β -spectrin, β' : mutated β -spectrin, * missing phosphorylation in the proband.



self-association of spectrin heterodimers in tetramers. The spectrin dimer–tetramer interconversion requires a high activation energy, and is immobilized kinetically at around 0 °C [25]. Therefore, the percentage of SpD and SpT in the crude spectrin extract at 0 °C indicates the relative distribution of these spectrins in the red cell membrane *in situ* [26]. When any mutations are present within or near the $\alpha\beta$ -spectrin heterodimer self-association site, these mutations lead to increased SpD [27–29], which is normally $5 \pm 5\%$, in the crude spectrin extract at 0 °C.

Tryptic peptide mapping of spectrin followed by electrophoretic separation is the third step to be performed (Fig. 11.4). When spectrin is partially digested by trypsin at 0 °C, and the resulting peptides are separated by two-dimensional isoelectric focusing-SDS–PAGE, reproducible peptides are obtained [30, 31]. There are five trypsin-resistant domains on the α -spectrin (α I through α V), and four on the β -chain (β I through β IV). Among these tryptic peptides of spectrin, the 80 kDa α I domain peptide represents the self-association site of the normal α -spectrin. Analysis of

**Figure 11.3** Dimer to tetramer conversion of purified spectrins.

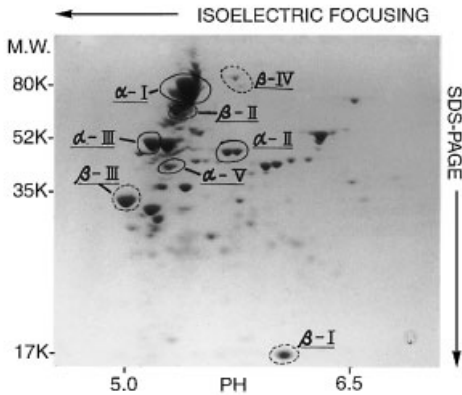


Figure 11.4 Normal spectrin peptides generated by limited tryptic digestion on two dimensional isoelectric focusing/sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). M. W: molecular weight.

spectrin isolated from patients with HE or HPP by this method often shows abnormal peptide fragments that appear at various positions when compared with the normal, due to the generation of new, abnormal tryptic cleavage sites in the mutant spectrin chains. Nearly all α - or β -spectrin mutations known are associated with the formation of tryptic peptides that are abnormal in size and mobility, which are replaced instead of the normal 80 kDa α I domain peptide (Fig. 14.1 B). The most common abnormal tryptic peptides demonstrate the cleavage sites which are located in the third helix of a given triple helical repetitive segment. The known mutations are located near these cleavage sites either in the third helix or, less commonly, in the first helix or the second helix of a given repetitive segment. Thus, tryptic peptide mapping is an important method for the detection of the site of the underlying spectrin mutation, which can be identified by amplification with a polymerase chain reaction (PCR) and sequencing of the respective region of the genomic DNA or cDNA, which are obtained from reticulocytes in peripheral blood.

11.3.3

Molecular Etiology

Elucidation of the molecular pathophysiology in HE or HPP has been achieved by DNA analysis of the genes of the major membrane skeletal proteins. The defects in HE or HPP appear to lie chiefly in the components of the red cell membrane skeleton that are responsible for the horizontal interactions within the skeletal network, especially, α -spectrin, β -spectrin and protein 4.1, along with glycophorin C as an integral protein.

First of all, most of the α -spectrin mutations lie in the N-terminal, α I domain that forms a region of the spectrin self-association site (Fig. 1.5). In most cases, the abnormal cleavage sites that produce the variant peptides are not the primary defects but are due to conformational changes of the spectrin chains associated with these defects. Generally speaking, there is an inverse relationship between the distance of the defect from the spectrin dimer self-association site and its asso-

ciated clinical severity. The closer to the self-association site, the greater the functional defect, and the more severe the clinical illness. In the majority of α -spectrin mutations, the mutations alter tryptic maps and lie close to the site of an abnormal tryptic cleavage site. Most are located in helix B (or helix C) of the spectrin repeats and appear to disrupt the triple helical structure of the repeats.

Numerous α -spectrin mutations have been reported in HE or HPP (Table 11.1). (1) The $\alpha^{1/78}$ defect indicates the presence of amino acid substitutions in codon 41 or 45 of the α -spectrin peptide [32–34]. (2) The $\alpha^{1/74}$ defect comes from heterogeneous mutations in codons 28, 34, 46, 48, or 49 [35–41]. Of these, codon 28, which contains a CpG dinucleotide site, appears to be a hot spot mutation, and is associated with four different mutations. The mutations in codons 45 or 48 are observed in severe HPP, and those in codons 41, 46, or 49 in a milder HE. (3) The $\alpha^{1/65}$ defect is associated with the duplication of a leucine residue in codon 154, or a missense mutation in codon 151, which is found in a mild HE [42–44]. (4) The $\alpha^{1/61}$ defect is yet to be examined. (5) The $\alpha^{1/50}$ defect comes either from substitution of a proline, which probably impairs α -helix formation [42, 45–47], or from a single residue deletion in codon 469 [48]. Amongst the $\alpha^{1/50}$ defects, a 49 residue deletion in codons 178 to 226 is found in spectrin Dayton [49]. (6) The $\alpha^{1/36}$ defect arises from deletion of codons 363 to 371 due to activation of a cryptic splice site, which is observed in spectrin Sfax [50]. (7) The $\alpha^{11/31}$ defect is based on a point mutation in codon 791 (spectrin Jendouba) [51]. (8) The $\alpha^{11/21}$ defect is associated with a deletion of codons 822 to 862 (spectrin Oran) from a mutation in the acceptor splice site for exon 18, resulting in the skipping of exon 18 [52].

Table 11.1 A list of gene mutations in hereditary elliptocytosis.

I. Mutations of the red cell α -spectrin gene (SPTA) in hereditary elliptocytosis

Name	Codon	Nucleotide	Protein	Mutation	Peptide (Repeat)
Lograno	24	ATC→AGC	I24S	Missense	–
Unnamed	28	CGT→TGT	R28C	Missense	$\alpha^{1/74}$ (1)
Corbeil	28	CGT→CAT	R28H	Missense	$\alpha^{1/74}$ (1)
Unnamed	28	CGT→CTT	R28L	Missense	$\alpha^{1/74}$ (1)
Unnamed	28	CGT→AGT	R28S	Missense	$\alpha^{1/74}$ (1)
Marseille	31	GTG→GCG	V31A	Missense	–
Genova	34	CGG→TGG	R34W	Missense	$\alpha^{1/74}$ (1)
Tunis	41	CGG→TGG	R41W	Missense	$\alpha^{1/78}$ (1)
Clichy	45	AGG→AGT	R45S	Missense	$\alpha^{1/78}$ (1)
Anastasia	45	AGG→ACG	R45T	Missense	–
Culoz	46	GGT→GTT	G46V	Missense	$\alpha^{1/74}$ (1)
Unnamed	48	AAG→AGG	K48R	Missense	$\alpha^{1/74}$ (1)

I. Continued.

Name	Codon	Nucleotide	Protein	Mutation	Peptide (Repeat)
Lyon	49	CTT→TTT	L49F	Missense	$\alpha^{1/74}$ (1)
Ponte de Sor	151	GGT→GAT	G151D	Missense	$\alpha^{1/65}$ (2–3)
Unnamed	154–155	3 nt ins (+TTG)	+L	Insertion	$\alpha^{1/65}$ (2–3)
Dayton	178–226	Insertion in intron 4	49 amino acids del.	Deletion	$\alpha^{1/50a}$ (3)
Saint Louis	207	CTG→CCG	L207P	Missense	$\alpha^{1/50a}$ (3)
Nigerian	260	CTG→CCG	L260P	Missense	$\alpha^{1/50a}$ (3–4)
Unnamed	261	TCC→CCC	S261P	Missense	$\alpha^{1/50a}$ (3–4)
Sfax	363	1086 A→G	9 amino acids del.	Abnormal splicing (Deletion)	$\alpha^{1/36}$ (4–5)
Alexandria	469	CAT→Del	1 amino acid (H469) del.	Deletion	$\alpha^{1/50b}$ (5–6)
Barcelona	469	CAT→CCT	H469P	Missense	$\alpha^{1/50b}$ (5–6)
Unnamed	471	CAG→CCG	Q471P	Missense	$\alpha^{1/50b}$ (5–6)
Jendouba	791	GAC→GAA	D791E	Missense	$\alpha^{11/31}$ (8–9)
Oran	822	2465–1 G→A	41 amino acids del.	Abnormal splicing (Deletion)	$\alpha^{11/21}$ (9)
St Claude	936	2806–13 T→G	31 amino acids del.	Abnormal splicing (Deletion)	$\alpha^{11/47}$ (10)
LELY	2177	6528–12 C→T	6 amino acids del. (exon 46)	Abnormal splicing (Deletion)	$\alpha^{V/41}$

II. Mutations of the red cell β -spectrin gene (*SPTB*) in hereditary elliptocytosis

Name	Codon	Nucleotide	Protein	Mutation
Prague	2008	6023–1 G→C	PCT	Abnormal splicing
Campinas	2008	6219+1 G→A	PCT	Abnormal splicing
Göttingen	2008	6219+2 T→A	PCT	Abnormal splicing
Le Puy	2008	6219+4 A→G	PCT	Abnormal splicing
Yamagata	2008	6219+4 A→G	PCT	Abnormal splicing
Kuwaitino	2018	Ala→Asp	A2018D	Missense
Cagliari	2018	GCC→GGC	A2018G	Missense
Providence	2019	TCT→CCT	S2019P	Missense
Paris	2023	GCG→GTG	A2023V	Missense
Linguere	2024	TGG→AGG	W2024R	Missense

II. Continued.

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>
Buffalo	2025	CTG→CGG	L2025R	Missense
Tandil	2041	6124–6130 7 nt del (–GACAGTG)	PCT	Frameshift (Del)
Nice	2046	6136–6137 2 nt ins (+GA)	PCT	Frameshift (Ins)
Kayes	2053	GCT→CCT	A2053P	Missense
Napoli	2053	6160–6167 8 nt del	PCT	Frameshift (Del)
Tokyo	2059	6177 1 nt del (–C)	PCT	Frameshift (Del)
Cotonoru	2061	TGG→AGG	W2061R	Missense
Cosenza	2064	CCC→CGC	R2064P	Missense
Nagoya	2069	GAG→TAG	E2069X	Nonsense
Rouen	2074	6269+3 G→T	PCT	Abnormal splicing

III. Mutations of the red cell protein 4.1 gene (*EL 1*) in hereditary elliptocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>
Algeria	1	318 nt deletion	Not expressed	Abolition of initiation codon
Annery	1	70 kb deletion	Not expressed	Abolition of initiation codon
Lille	1	ATG→ACG	M1T	Missense
Madrid	1	2 ATG→AGG	M1R	Missense
Unnamed	407	Lys 407–Gly 486 deletion	K407–G486 deletion	Deletion
Hurdle-Mills	407	Lys 407–Gln 529 deletion	K407–Q529 duplication	Duplication
Aravis	447	Lys deletion	K447 deleted	Deletion

IV. Mutation of the red cell anion exchanger-1 (*AE1*: band 3) gene (*EPB3*) in hereditary elliptocytosis

<i>Name</i>	<i>Codon</i>	<i>Nucleotide</i>	<i>Protein</i>	<i>Mutation</i>
Southeast	56	AAG→GAG	Lys56Glu	Polymorphism
Asian ovalocytosis (SAO)	400–408	27 nt deletion	Deleted (400Ala– 408Ala)	Deletion

It is interesting to note that there are very few truncated α -spectrin peptides in HE or HPP, whereas most of the β -spectrin abnormalities are associated with serious gene mutations such as frameshift mutations, nonsense mutations, or abnormal splicings, leading to premature chain termination. It should be considered that α -spectrin is synthesized excessively, three- to four-fold more than β -spectrin during erythroid development and maturation. Therefore, even if α -spectrin mutation was to be present in one allele, enough α -spectrin chains from another normal allele would still be produced to make complete forms of α - and β -spectrin tetramers. Thus, one may expect that heterozygous individuals are completely asymptomatic and have their red cells of normal biconcave disc shape, in contrast to homozygous individuals with severe hemolytic anemia, poikilocytosis, microspherocytosis, and elliptocytosis.

As described previously, the closer to the self-association site, the greater the functional defect, and the more severe the clinical phenotype. The best examples are the $\alpha^{I/74}$ defects, which are located right at the spectrin dimer self-association site, are accompanied by severe hemolytic anemia with a markedly increased SpD even in heterozygotes. The $\alpha^{I/65}$ defects, in contrast, which are located further from the self-association site, demonstrate milder hemolysis and impaired spectrin self-association but to a lesser extent. The situation is much more evident in the $\alpha^{II/21}$ defect, which lies a long distance from the self-association site. Under these conditions, the illness is very mild even in homozygotes.

It is known that in a common HE kindred with α -spectrin mutations, some family members may develop hemolytic anemia which is disproportionately severe compared with that expected from this type of primary mutation. In this case, the presence of a second, low-expression allele is suspected, which is silent in carriers but contributes to the severity of the disease when present *in trans* to a structurally mutant allele. The low-expression α -spectrin allele is known as α^{LELY} (Low Expression allele LYon) [53]. The best example is the co-existence of the α -spectrin variant ($\alpha^{\text{V/41}}$) and a C-to-T substitution in an acceptor splice site 12 nucleotides before the splice junction that leads to 50% in-frame skipping of exon 46, which is only 18 base pairs in length [54–56]. The exon lies within the nucleation site for α - to β -chain association. α -Spectrin chains lacking exon 46 fail to bind to β -spectrin chains or to be incorporated into the membrane skeleton, and are instead destroyed, because the nucleation site is interfered with under these conditions. The function of the remaining α -spectrins is normal, because the exon 46 is present. The α^{LELY} allele itself is completely silent even in homozygous individuals, because α^{LELY} splicing mutation causes only partial skipping of exon 46 and, furthermore, α -spectrin peptides are produced in excess even under normal conditions. When the α^{LELY} allele is present *in trans* to a second allele encoding a structurally abnormal α -spectrin associated with HE, the mutant α -spectrin peptide with the structural defect will be incorporated preferentially into the membrane. As a result, the severity of the disease is aggravated drastically. When an HE mutation is present on a chromosome bearing the α^{LELY} mutation *in cis*, a mild defect can be converted into a silent one or a severe one into a mild one. On the other hand, when a mutated HE allele is present *in trans* to the α^{LELY} allele, a more severe clinical condition may develop such as severe HE or HPP. The α^{LELY} allele is found in a sur-

prisingly high frequency (16–31 %) in all ethnic groups such as Caucasians, Africans, Japanese, Chinese, and Amazon Indians [57].

Now considering β -spectrin mutations, more than 20 mutations in β -spectrin have been identified in HE or HPP. The mutation sites are strictly clustered at or near the spectrin-dimer self-association site at the C-terminus of β -spectrin (Fig. 11.5). The types of mutations are various such as exon skipplings [58–62], frameshift mutations [63–67], nonsense mutations [68] as well as missense mutations [35, 69–75] (Table 11.1). For example, in spectrin Tokyo ($\beta^{220/216}$) [63], which is one of the β -spectrin truncated variants, about 4 kDa are lost from the C-terminus of β -spectrin due to a frameshift mutation (GCCAGC→GCAGCT) in codon 2059 of the β -spectrin gene. Low temperature spectrin extracts contain increased spectrin dimer (27 % of the total amount of the spectrin dimer–tetramer) compared with normal (5 %), and most of the abnormal truncated β^{216} spectrin is observed to be in the dimer fraction (Fig. 14.4). Detailed information is available in Sections 4.1.2 and 14.2.2. It is clearly indicated that the truncated β^{216} peptide is incapable of oligomerizing properly (Fig. 14.4), and that this inability is responsible for the functional defect of β -spectrin. In spectrin Tokyo ($\beta^{220/216}$), the same as with other β -spectrin mutations, α -spectrin abnormality (such as $\alpha^{1/74}$) is also observed by tryptic mapping. This is understood to be the result of a secondary abnormality, in which the N-terminus of the α -spectrin peptide is unable to bind to the truncated C-terminus of the β -spectrin peptide.

It is interesting to note that the longer the region of the truncated peptide at the N-terminus of β -spectrin, the greater the functional defect, and the more severe the clinical phenotype. This tendency is clearly observed among three types of β -spectrin anomalies, that is: β -spectrin Le Puy in Yamagata ($\beta^{220/214}$) [62], β -spectrin Tokyo ($\beta^{220/216}$) [63], and β -spectrin Nagoya ($\beta^{220/217}$) [68], in which the last normal codons are 2007, 2059, and 2069, respectively, as discussed in Sections 14.2.2, 14.2.3, and 14.2.4 (Fig. 11.5).

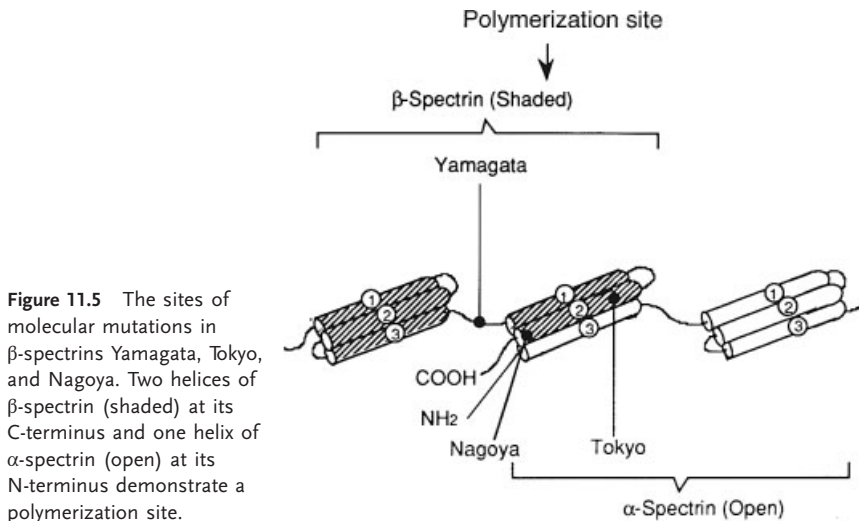


Figure 11.5 The sites of molecular mutations in β -spectrins Yamagata, Tokyo, and Nagoya. Two helices of β -spectrin (shaded) at its C-terminus and one helix of α -spectrin (open) at its N-terminus demonstrate a polymerization site.

Protein 4.1 mutations have also been reported in HE.

Quantitative and qualitative defects of protein 4.1 due to protein 4.1 mutations have also been reported in HE. As regards quantitative abnormalities of protein 4.1, four independent families have been known to have total deficiency of protein 4.1, i. e.: protein 4.1 Algeria [76], protein 4.1 Annery [77], protein 4.1 Lille [78], and protein 4.1 Madrid [79] (Table 11.1). Alternate translation initiation sites are normally present in the protein 4.1 mRNA (Figs. 4.5 and 14.10). When an upstream AUG is utilized, isoforms greater than 80 kDa are synthesized. During erythropoiesis, this upstream AUG is spliced out and a downstream AUG is utilized, leading to the production of the 80 kDa mature erythroid protein 4.1 isoform. In these mutations of the protein 4.1 gene, the downstream initiation codon, which should be utilized by the 80 kDa erythroid isoform of protein 4.1, is eliminated by a 318 base pair deletion in protein 4.1 Algeria [76], a 70 kb deletion in protein 4.1 Annery [77], a missense mutation of MIT in protein Lille [78], and a missense mutation of M1R in protein 4.1 Madrid [79], respectively. In protein 4.1 Aravis [80], deletion of a single residue (K 447 del) in the spectrin-binding domain eliminates its ability to bind spectrin. Detailed information on protein 4.1 Madrid is available in Section 14.3.3 (Figs. 14.9–14.15). These homozygous protein 4.1 (–) red cells also lack p55 and have only 30 % of the normal content of glycophorin C. Electron microscopic studies of homozygous 4.1 (–) Madrid red cell membranes revealed a markedly disrupted skeletal network with disruption of the intramembrane particles [81], suggesting that protein 4.1 plays an important role in maintenance not only of the skeletal network, but also of the integral proteins of the membrane structure.

Qualitative defects of protein 4.1 include deletions and duplications of the exons encoding the spectrin-binding domain, leading either to truncated [82–84] or elongated [83, 84] forms of protein 4.1. The shorter form (protein 4.1^{68/65}) is derived from a deletion of 240 base pairs (Lys⁴⁰⁷–Gly⁴⁸⁶) of the protein 4.1 gene, which include the entire 10 kDa spectrin–actin binding domain, resulting in severe disruption of the cytoskeletal network [82–84]. However, the elongated form (protein 4.1⁹⁵) is produced by duplication of the segment of Lys⁴⁰⁷–Gln⁵²⁹, which includes the entire 10 kDa spectrin–actin binding domain, in the protein 4.1 gene of the patient [83, 84]. The clinical phenotype is a mild HE without anemia, probably because the essential spectrin–actin binding domain is well preserved. Protein 4.1 Presles is a third variant with a shortened protein 4.1 that migrates as a doublet with apparent sizes of 73 and 74 kDa [85]. This anomaly is caused by skipping one exon that encodes 34 amino acids near the beginning of the C-terminal 22/24 kDa domain [86]. The homozygous patient is clinically silent.

Although a partial deficiency of protein 4.1 appears to be present in numerous patients with mild dominantly inherited HE, the genetic pathogeneses have not been well defined because of the extremely complicated splicing events and polymorphisms of the protein 4.1 gene in human beings (see Section 4.2 and Fig. 4.5). Most patients with HE in Japan appear to be associated with partial deficiencies of protein 4.1 [9].

Glycophorin C deficiency is also associated with elliptocytosis, especially the so-called Leach phenotype, which is caused by reduced expression of glycophorin C

(see Sections 5.2.2, 15.2.2, and Fig. 15.18). The Leach phenotype is usually due to a large deletion (exons 3 and 4) of genomic DNA of glycophorin C and D. Glycophorin C-deficient individuals are accompanied with a partial deficiency of protein 4.1 and a lack of p55. This fact suggests that glycophorin C may form a complex with protein 4.1 and p55, or that they stabilize each other on the membrane. The red cell morphology in glycophorin C deficiency of the Leach type demonstrates elliptocytosis, whereas that in glycophorin A deficiency does not, having the normal biconcave disc shape.

Band 3 abnormalities have not been detected in HE/HPP, except for Southeast Asian ovalocytosis (SAO) [11, 18–23], in which two genetic abnormalities are present, that is, band 3 Memphis (K56E), and deletion of amino acid residues 400–408 at the junction between the cytoplasmic and membrane domains [87, 88]. This deletion removes part of the first transmembrane α helix. Consequently, band 3 SAO lacks anion transport activity. SAO red cells are known to be extremely rigid [89]. The causal relationship between this extreme rigidity and the gene deletion of codons 400–408 has not been well defined. The pathogenesis of the formation of ovalocytosis in this disorder also remains to be elucidated at some point in the near future.

11.4

Hereditary Pyropoikilocytosis (HPP)

Hereditary pyropoikilocytosis (HPP) is a rare disorder in infancy or early childhood, which manifests itself as severe hemolytic anemia with a hemoglobin level of from 2 to 5 g dL⁻¹ characterized by extreme poikilocytosis with budding red cells, fragmented red cells, spherocytes, elliptocytes, triangular cells, and other bizarre-shaped red cells [10, 15, 16]. The first case was reported by Zarkowsky et al. in 1975 [10]. Although this HPP was initially considered as a separate entity, there is much convincing evidence that this disorder is related to HE. HPP is clinically and morphologically similar to the more severe forms in homozygous HE and HE with poikilocytosis in infancy. In many HPP cases, one of the parents or siblings has typical mild common HE. In some of these kindred, an identical molecular defect is found in siblings with phenotypically different diseases. A normal phenotype can also be observed in all of the first-degree relatives in other families. Hemolytic anemia is usually severe, requiring acute or chronic transfusion. In addition to severe anemia, growth retardation, frontal bossing, and early gallbladder disease may also be complications. Most of the cases have been reported in individuals of African origin. Clinically, HPP patients present with hyperbilirubinemia in the neonatal period or with severe anemia in the first few months of life. Red cell fragmentation, erythroblastosis, and splenomegaly have been reported.

A number of biochemical and molecular defects are shared between HE and HPP. Laboratory tests reveal very abnormal osmotic fragility tests (especially after incubation for 24 h), and markedly increased autohemolysis. The mean corpuscular volume (MCV) is very low (40–60 fL) in severely affected patients due to marked red cell fragmentation.

The identifying feature of this disorder is a remarkable thermal sensitivity of the affected red cells [10]. The HPP red cells fragment at 45–46 °C after short periods of heating (10–15 min), compared with 49 °C in normal red cells (Fig. 14.1 A). With prolonged heating of longer than 6 h, the HPP red cells may fragment even at 37 °C.

Another characteristic feature is a marked deficiency of spectrin in HPP red cells (up to 30 % less than the normal quantity) [17]. HPP red cells, but not HE red cells, are usually markedly deficient in spectrin. This associated spectrin deficiency may explain why HPP patients often demonstrate phenotypes observed in HS patients, such as the presence of microspherocytosis and an abnormal osmotic fragility. In typical HPP, one parent of the HPP offspring carries an α -spectrin mutation whereas the other parent is fully asymptomatic and has no detectable biochemical abnormality. Studies of spectrin synthesis and mRNA levels present show that such asymptomatic parents carry a silent thalassemia-like defect of spectrin synthesis. In the case where this thalassemia-like defect is inherited coincidentally with the elliptocytogenic spectrin mutation in the HPP offspring, this defect enhances the expression of the mutant spectrin in the cells and leads to a superimposed spectrin deficiency.

HPP patients are basically heterozygous for a structural variant of spectrin involving the self-association site but have a more severe phenotype than expected. It has been postulated that they have a second defect of α -spectrin that affects spectrin production or accumulation. The parents who transmit the postulated defect are clinically and biochemically normal.

A typical example is the low expression allele of α -spectrin (the α^{LELY} allele) [53]. Cases where HPP patients are heterozygous for various mutant α -spectrins and who have the $\alpha^{\text{V}/41}$ polymorphism *in trans* are more severe than expected. However, the polymorphism itself is asymptomatic in either the heterozygous or homozygous state. Molecular studies identified two linked abnormalities. Together these changes identify the α^{LELY} allele, which has a wide ethnic distribution and is very common. In patients who are heterozygous for α^{LELY} and α -spectrin mutation causing HPP, the limited synthesis of α^{LELY} protein decreases the amount of spectrin containing α^{LELY} that is incorporated into the membrane by around 50 % and increases the relative incorporation of spectrin containing the HPP α -chain. Alpha spectrin chains that lack exon 46 fail to assemble into stable spectrin dimers and are degraded owing to defective spectrin nucleation. Spectrin α^{LELY} should be distinguished from the thalassemia-like defects of α -spectrin synthesis that also produce a phenotype, when they are co-inherited with some of the α -spectrin mutations. Thalassemia-like defects are characterized by reduced α -spectrin mRNA levels and diminished α -spectrin synthesis.

Splenectomy is effective, and hemolysis is markedly reduced after splenectomy, but not eliminated, giving for example, a 10–14 g dL⁻¹ hemoglobin level and 3–10 % reticulocytes in peripheral blood.

11.5

Southeast Asian Ovalocytosis (SAO)

In the 1970s it was reported that a unique type of ovalocytosis of hereditary origin existed in the aboriginal populations of Melanesian and Malaysia and also in some regions of Indonesia and the Philippines [11, 18, 19]. In lowland tribes, 12.2–22.4 % of the inhabitants are affected. This region is known to be where malaria is endemic. The prevalence of hereditary elliptocytosis, that is, Southeast Asian ovalocytosis (SAO) increases with age in this population. SAO appears to protect against malaria infections, especially serious infections and cerebral malaria. Therefore, individuals who have SAO appear to have a selective advantage in life.

The morphological characteristic of SAO red cells is rounded elliptocytosis (ovalocytosis) with stomatocytic features. The extent of hemolysis is mild or absent, and anemia by hemolysis is apparently fully compensated. The red cell membranes of this disorder demonstrate unusually high heat resistance [22]. The maximum temperature for denaturation of red cell membranes is 52 °C in this disorder compared with 49 °C in normal red cells and with 46 °C in hereditary pyropoikilocytosis (HPP). Other characteristic features of the SAO red cells are a lack of drug-induced endocytosis, resistance to crenation after storage in plasma or buffered saline solutions, increased Na⁺ and K⁺ permeability, elevated glucose consumption compensating for increased cation pumping, and increased autohemolysis [21]. It should be noted that the SAO red cells are osmotically resistant rather than fragile [22]. In addition, in the SAO red cells, many blood group antigens are poorly expressed or even missing such as I^T, I^F, LW, D, C, e, S, s, U, Kp^b, Jk^a, Jk^b, Xg^a, Wr^b, Scl, and En^a [23]. The pathogenesis is yet to be elucidated.

Through molecular biology, it has been discovered that all carriers of the SAO phenotype are heterozygous for two mutations *in cis*: a deletion of nine codons encoding amino acids 400 through 408 [88, 89], which are located at the boundary of the cytoplasmic and membrane domains and the missense mutation of Lys⁵⁶→Glu⁵⁶ (an asymptomatic polymorphism as band 3 Memphis I) [87]. This deletion partly removes the first transmembrane α helix as an internal signal sequence, resulting in disruption of the structure of the membrane domain [90]. In addition, band 3 SAO is deficient in anion transport function [91]. In these carriers, one band 3 allele is normal, and the other allele contains two mutations as described above.

There appears to be no SAO homozygotes that are lethal. SAO is caused by deletion of 27 bases from the band 3 gene which produces a single band in normal red cells and a doublet with the second band shorter, by 27 base pairs, in SAO red cells.

Biochemically, the mutated SAO band 3 demonstrates marked restriction of lateral and rotational mobility, impaired sulfate anion transport [91], increased tyrosine phosphorylation, and tight binding to ankyrin. SAO red cells are resistant to malaria invasion *in vitro*, probably because the SAO red cell membrane is 10 to 20 times more rigid than normal. The rigidity of SAO red cells appears to be attributed to the impaired lateral movement of the skeletal network during red

cell deformation due to conformational changes of the cytoplasmic domain of the mutated band 3 of SAO red cells [92]. In addition to the tendency of the SAO band 3 to bind abnormally tightly to the underlying skeleton through increased binding to ankyrin, the SAO band 3 tends to aggregate into higher oligomers, which may enhance band 3 attachment to ankyrin. Thus, the SAO band 3 adheres to the skeleton firmly.

The characteristic feature of SAO red cells is their resistance to malaria infections [21]. Band 3 is known to be one of the malaria receptors, and parasites invade red cells with marked membrane remodeling and redistribution of intramembrane particles (IMPs) including band 3. In normal red cells, IMPs cluster at the sites of malaria invasion on red cell membranes in the form of a ring around the entrance orifice of this invasion. In SAO red cells, attachment and entry of malaria may be blocked by the reduced lateral mobility of band 3 and impaired band 3 receptor clustering [92]. Decreased exchange of anions across the SAO red cell membrane [91] may also contribute to the resistance of ovalocytes to malaria invasion. Exposure of red cells to various ligands that bind to glycophorin A has been found to decrease membrane deformability.

The most specific test for establishing the diagnosis of SAO is the isolation of genomic DNA or reticulocyte cDNA with subsequent amplification of the deletion-containing region, because the underlying cause of SAO is basically the deletion of 27 bases from the band 3 gene (Fig. 11.6). Another useful screening test is the clinical demonstration of the resistance of ovalocytes or their ghosts to changes in shape produced by treatments that produce spiculation in normal cells, such as metabolic depletion (prolonged incubation of the SAO ovalocytes without glucose) or exposure of red cell ghosts to salt solutions.

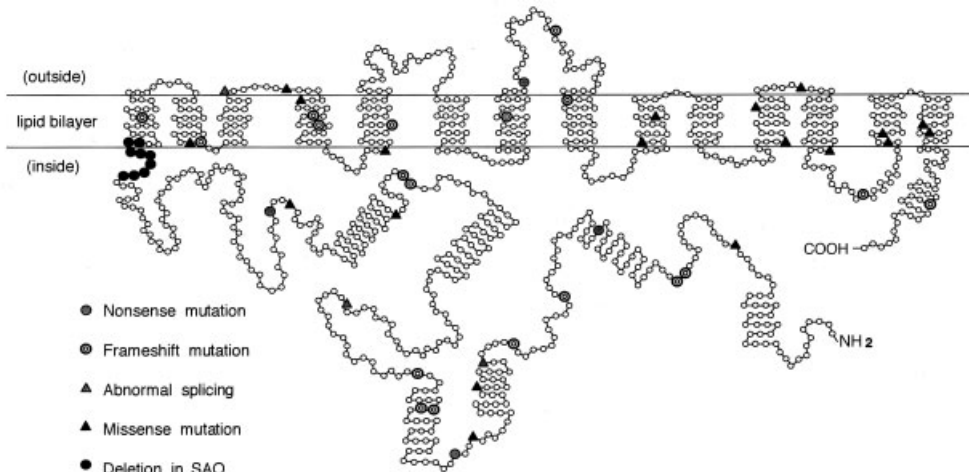


Figure 11.6 Schematic demonstration of the localization of mutations of the human band 3 gene.

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12

Hereditary Stomatocytosis

12.1

Introduction

Stomatocytosis is a syndrome of various disorders of heterogeneous origins with stomatocytes, which are characterized by a wide transverse slit or stoma in the red cell smear from peripheral blood [1–7].

The extent of stomatocytic change varies from discocytes through disco-stomatocytes, stomatocytes, and stomatospherocytes, to sherocytes (Fig. 2.4). The characteristic red cell shape can be investigated in a wet film better than in a dry blood smear (Fig. 12.1). The red cell morphology can be well preserved by fixing red cells with isotonic phosphate buffer containing 1 % glutaraldehyde solution. One can reexamine the red cell morphology repeatedly anytime under light microscopy or scanning electron microscopy, once the red cells are fixed.

Normal red cells of biconcave disc shape can be transformed by modifying their skeletal proteins or the lipid bilayer of the membrane [8–19]. As protein modifiers, vinca alkaloids (especially 0.3 mM vinblastine for a 2 h incubation), or colchicine

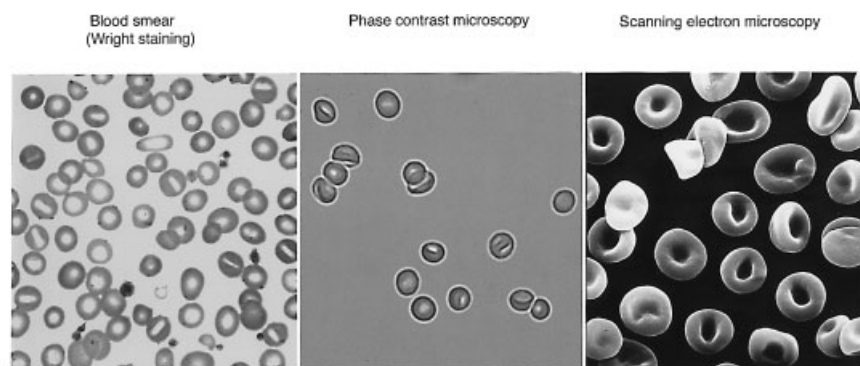


Figure 12.1 Morphological characteristics of hereditary stomatocytosis. Red cell morphology in a patient with hereditary hydrocytosis was examined by three different methods, that is, by blood smear (with Wright-staining; left), by phase contrast light microscopy (middle), and by scanning electron microscopy (right). It should be noted that the findings by phase contrast light microscopy are identical to those by scanning electron microscopy, which is known to be most suitable for observing the stereotactic shapes of red cells *in vivo*.

(9 mM for a 2 h incubation) can produce stomatocytic changes in more than 80% of normal red cells. Lecithin (4.2% for 5 h), cholic acids (2 mM for 1 h), Triton X (0.02% for 2 h), and primaquine (0.9 mM for 4 h) are also effective at producing stomatocytes, probably by interacting with the membrane lipid bilayer of normal red cells. These results may indicate that molecular modifications either of membrane proteins or of membrane lipids are able to induce red cell shape changes such as stomatocytosis. It has also been shown that when membrane lipids in normal red cells are treated with phospholipase A₂, the modification induces an increase in sodium leakiness, in addition to the formation of stomatocytes [20].

Concomitant to this stomatocytic change which is induced by various molecular modifiers, the monovalent cation transport (sodium influx) is substantially increased: most markedly (three-fold that of the normal discocytes) in vinblastine, moderately (roughly two-fold) in primaquine and lecithin, and least (1.1–1.5-fold) in cholic acids and colchicine even with the same degree of stomatocyte formation.

The abnormalities of cation transport are usually expected to be associated with the abnormalities of cell hydration.

Since the first description of hereditary stomatocytosis by Lock et al. (1961) [21], a variety of cases have been reported. From the standpoint of abnormal cell hydration, the features of this disorder are fairly heterogeneous as would be expected from the experimental data, including the greatly increased cell hydration (hydrocytosis) at one pole and the decreased cell hydration (dehydrocytosis, xerocytosis, or desiccytosis) at the other pole (Fig. 12.2). Thus, hereditary stomatocytosis is indeed a syndrome in the presence of marked stomatocytosis. The exact pathogenesis of this disorder has not been clarified, except for the presence of rare cases with red cell membrane lipid abnormalities such as hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCA) [22] and congenital lecithin:cholesterol acyltransferase deficiency [23]. Abnormalities of red cell membrane lipids do induce stomatocytosis with impaired sodium transport (see Chapter 17).

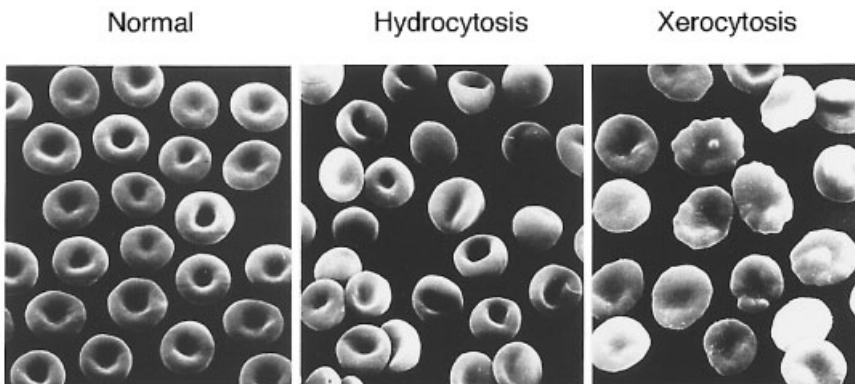


Figure 12.2 Scanning electron micrographs of red cells in hereditary stomatocytosis.

Thus, it is preferable to discuss the cases with stomatocytosis that are associated with distinct plasma and/or red cell membrane lipid abnormalities under the category of red cell membrane lipid disorders (Chapter 17). In the present chapter, hereditary stomatocytosis with normal membrane lipids will be described.

Throughout our studies of 44 patients with stomatocytosis [7], we have become aware of the presence of a fair number of patients (22 cases) with marked stomatocytosis, but without any impaired sodium transport. In addition, approximately one-third of the patients with stomatocytosis did not show any overt hemolysis [7]. In general, there was no correlation between the extent of sodium influx and the extent of hemolysis [7]. Even in some patients with striking stomatocytosis, normal sodium influx was observed. Since various levels of sodium influx were observed in the stomatocytosis groups, abnormal sodium transport *per se* cannot be the single determinant for red cell shape change [7].

It has been reported that red cell membrane protein 7.2b consists of three components (30, 28 and 26 kDa), covering approximately 3.4% of the total ghost proteins [24]. A deficiency of the 28 kDa integral protein was reported in patients with hydrocytosis and cryohydrocytosis. This observation is confirmed only in some, but not all, patients with stomatocytosis, mainly hydrocytosis [7]. Thus, it appears that the deficiency of protein 7.2b is not specific to hereditary stomatocytosis [25].

Stomatocytosis is also observed in individuals with a deficiency of the blood group antigen Rh (Rh null) [26] or with cold hemolysis (cryohydrocytosis) [27].

Acquired stomatocytosis [28] has been reported in the patients with neoplasms, cardiovascular and hepatobiliary disease, alcoholism [29] and therapy with drugs (especially vinca-alkaloids [30] such as vinblastine), in which the clinical course is transient.

12.2

Hereditary Hydrocytosis

This disorder is characterized by a dominantly inherited hemolytic anemia with increased red cell hydration and macrocytosis [1–6]. The first report was made by Lock et al. in 1961 [21], and then Zarkowsky et al. [31] elucidated the presence of abnormal cation transport and cellular overhydration in this disorder.

The identifying feature of this disorder lies in a sodium leak leading to an increase in red cell sodium and water content with a mildly decreased potassium content in the red cells [1–7]. Concomitant to this increased sodium leak into the red cells, is a compensatory increase in the active transport of sodium and potassium by the Na^+/K^+ -ATPase pump, through which the low intracellular sodium and high potassium content are intended to maintain normality [32]. Although increased glycolysis is usually accompanied by these functional abnormalities, the markedly increased sodium leak is not compensated for. The exact molecular mechanism of this remarkably increased permeability in the patient's red cells has not been elucidated.

It has been reported that protein 7.2 in red cells consists of three components (30, 28, and 26 kDa), covering approximately 3.4% of the total ghost membrane

proteins. A major component (28 kDa) migrated to the basic side as an integral protein and to the acidic side as a peripheral protein by two-dimensional nonequilibrium pH gradient electrophoresis (NEPHGE)/SDS–PAGE.

In the red cells of some patients with hereditary hydrocytosis, the lack of an integral protein (protein 7.2b or stomatin) was found [24]. The degree of stomatin deficiency varied. In addition, the deficiency was observed in some but not all patients of hereditary stomatocytosis [7, 33]. In our experience, a partial deficiency of protein 7.2 was detected in three out of 44 patients with hereditary stomatocytosis including six patients with hereditary hydrocytosis [7]. The extent of protein 7.2 deficiency was essentially unrelated to the extent of sodium influx [7]. In addition, no appreciable abnormalities of protein 7.2 were detected in hereditary hydrocytosis [7]. Instead, a 30% reduction in a basic side component was observed in a case of hereditary xerocytosis rather than hydrocytosis [7]. The relative content (protein 7/protein 5 ratio) was $12.5 \pm 1.1\%$ in six patients with hereditary hydrocytosis, compared with $12.3 \pm 4.0\%$ in 20 normal individuals [7].

The stomatin cDNA from several patients with hereditary stomatocytosis was reported to be normal [34–37]. Mice lacking stomatin demonstrate no hemolytic anemia, normal morphology of red cells, normal red cell indices, and normal cation content with normal cell hydration [25]. Therefore, the defect in stomatin appears not to be the primary defect in hereditary stomatocytosis but might be involved in an as yet unelucidated mechanism for a cellular volume regulatory pathway in the red cells.

In clinical hematology, hereditary hydrocytosis exhibits moderate to severe anemia. In our experience in the six patients with hereditary hydrocytosis [7], the levels were $3.12 \pm 0.85 \times 10^{12} \text{ L}^{-1}$ in red cell count, $10.9 \pm 2.9 \text{ g dL}^{-1}$ in hemoglobin, $119.6 \pm 8.5 \text{ fL}$ in MCV (normal: 88.0 ± 5.5), $29.3 \pm 1.8\%$ in MCHC (normal: 34.5 ± 1.5), $16.1 \pm 11.7\%$ in reticulocytes, and $13 \pm 10 \text{ mg L}^{-1}$ in indirect bilirubin (normal: 3 ± 2). Jaundice and splenomegaly are usually observed, and cholelithiasis may be complicated. The peripheral blood smear indicates marked stomatocytosis. In our six patients with hereditary hydrocytosis, the extent of stomatocytosis was $54.8 \pm 10.7\%$ of the total red cells, including $13.6 \pm 6.4\%$ of disco-stomatocytes, $22.9 \pm 6.3\%$ of stomatocytes, $13.3 \pm 4.5\%$ of stomato-spherocytes, and $5.0 \pm 2.8\%$ of stomatocytic triconcave red cells [7]. Decreased MCHC ($29.3 \pm 1.8\%$) and elevated MCV ($119.6 \pm 8.5 \text{ fL}$) have been described previously. Red cell osmotic fragility is significantly increased.

Red cell membrane transport was clearly abnormal in our six patients with hereditary hydrocytosis [7]. Sodium influx was $8.90 \pm 3.39 \mu\text{mol L}^{-1}$ red cells per hour (normal: 1.29 ± 0.14), and sodium efflux was also significantly elevated at $10.16 \pm 3.92 \mu\text{mol L}^{-1}$ red cells per hour (normal: 2.40 ± 0.50), in which $3.48 \pm 0.97 \mu\text{mol L}^{-1}$ red cells per hour (normal: 1.40 ± 0.70) were the ouabain-sensitive sodium efflux, and $6.68 \pm 3.59 \mu\text{mol L}^{-1}$ red cells per hour (normal: 1.00 ± 0.30) were the ouabain-insensitive, respectively. Red cell sodium content was markedly elevated ($45.4 \pm 22.9 \text{ mM}$; normal: 10 ± 3), and red cell potassium was decreased significantly ($40.4 \pm 12.5 \text{ mM}$; normal: 90 ± 5) [7]. The activity of Na^{+} , K^{+} -ATPase in red cells was enhanced at $5.20 \pm 2.20 \text{ mmol L}^{-1}$ red cells per hour (normal: 2.52

± 0.60). Red cell membrane lipid analysis revealed essentially normal results in our six patients with hereditary hydrocytosis [7], that is: $1394 \pm 95 \mu\text{g}$ per 10^{10} red cells (normal: 1202 ± 103) of free cholesterol and $2651 \pm 300 \mu\text{g}$ per 10^{10} red cells (normal: 2604 ± 241) of total phospholipids. The fractions of phospholipids were also basically normal, that is: $27.8 \pm 2.3\%$ of phosphatidylethanolamine (normal: 30.9 ± 0.8), $16.2 \pm 2.4\%$ of phosphatidylserine and phosphatidyl-inositol (normal: 14.1 ± 0.9), $27.7 \pm 2.0\%$ of phosphatidylcholine (normal: 28.2 ± 1.0), $26.0 \pm 1.4\%$ of sphingomyelin (normal: 25.4 ± 1.2), and $4.3 \pm 4.7\%$ of lyso-phosphatidylcholine (normal: 1.5 ± 0.5), respectively.

Most patients with hereditary hydrocytosis suffer from life long hemolytic anemia, similar to those with hereditary spherocytosis. Although splenectomy is usually effective in hereditary hydrocytosis unlike hereditary xerocytosis, some hydrocytosis patients may develop serious life-threatening hypercoagulability which may result in venous thromboembolism [38]. Treatment of splenectomized patients with long-term coumarin administration may be beneficial. Neonates with hydrocytosis usually require phototherapy at birth. In some cases, exchange transfusion for the treatment of anemia and hyperbilirubinemia may be needed.

12.3

Hereditary Xerocytosis

Hereditary xerocytosis is also known as hereditary dehydrocytosis, or hereditary desiccocytosis because the patients' red cells are definitely dehydrated [1–5, 7, 39]. This disorder is an autosomal dominantly inherited hemolytic anemia associated with red cell dehydration and decreased osmotic fragility.

The exact pathogenesis of cell dehydration in this disorder has not been well elucidated, although the red cells demonstrate a net loss of potassium that is not accompanied by a proportional gain of sodium. Through this mechanism, the net intracellular cation content and cell water content are reduced. The chromosomal locus for hereditary xerocytosis is reported to be around 16q23–qter [40].

Hereditary xerocytosis should be critically differentiated from the diseases with red cell membrane lipid abnormalities, because these disorders, such as hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA) [22], congenital lecithin:cholesterol acyltransferase (LCAT) deficiency [23] and others, also demonstrate a dehydrated red cell syndrome due to membrane lipid abnormalities (see Chapter 17). No membrane lipid abnormalities are present in hereditary xerocytosis, in which membrane lipids themselves are not pathognomonic.

The classification of hereditary xerocytosis has not been well established, although several tentative titles have been postulated; cryohydrocytosis, stomatocytic xerocytosis, pseudohyperkalemia, and dehydrated stomatocytosis (oxyrocytosis in a narrower sense) [2]. Because molecular pathogenesis of this disorder has not been elucidated, the total picture is still confusing and unclarified.

Patients with hereditary xerocytosis usually suffer from uncompensated hemolytic anemia, jaundice, splenomegaly, and gallstones. In our experience with seven in-

dependent patients with hereditary xerocytosis [7], red cell count was $3.35 \pm 0.61 \times 10^{12} \text{ L}^{-1}$, hemoglobin $10.6 \pm 3.6 \text{ g dL}^{-1}$, MCV $91.0 \pm 6.2 \text{ fL}$, MCHC $34.7 \pm 1.6 \%$, reticulocytes $6.0 \pm 4.2 \%$, and indirect bilirubin $0.7 \pm 0.5 \text{ mg dL}^{-1}$. Blood films reveal moderate dehydrated stomatocytosis ($38.8 \pm 12.8 \%$; normal: 4.2 ± 2.7) and a few typical target cells ($1.2 \pm 0.3 \%$; normal: 0.2 ± 0.1) [7]. This observation may be important to differentiate hereditary xerocytosis with normal membrane lipids from HPCHA [22], in which there are many significant dehydrated stomatocytes ($89.7 \pm 8.8 \%$) and markedly increased target cells ($28.7 \pm 5.6 \%$) due to abnormally elevated free cholesterol and phosphatidylcholine in its red cell membranes.

Red cell membrane lipids in our seven patients with hereditary xerocytosis were essentially normal [7], that is, free cholesterol $1221 \pm 169 \mu\text{g per } 10^{10} \text{ red cells}$, and total phospholipids $2456 \pm 250 \mu\text{g per } 10^{10} \text{ red cells}$. Each fraction of phospholipids was also within the normal range, that is, phosphatidylethanolamine $725 \pm 80 \mu\text{g per } 10^{10} \text{ red cells}$ (normal: 806 ± 86) phosphatidylserine and phosphatidylinositol $333 \pm 36 \mu\text{g per } 10^{10} \text{ red cells}$ (normal: 366 ± 38), phosphatidylcholine $730 \pm 58 \mu\text{g per } 10^{10} \text{ red cells}$ (normal: 733 ± 64), sphingomyelin $639 \pm 70 \mu\text{g per } 10^{10} \text{ red cells}$ (normal: 663 ± 73), and lyso-phosphatidylcholine $29 \pm 1 \mu\text{g per } 10^{10} \text{ red cells}$ (normal: 39 ± 1).

The most critical feature in hereditary xerocytosis is cation transport abnormalities in the red cells [7]. Sodium influx was $2.10 \pm 0.82 \mu\text{mol L}^{-1} \text{ red cells per hour}$, and sodium efflux was $5.45 \pm 2.53 \mu\text{mol L}^{-1} \text{ red cells per hour}$. Red cell sodium content was $15.3 \pm 6.2 \text{ mM}$, and red cell potassium content was $82.9 \pm 7.4 \text{ mM}$. The activities of Na^{+} , K^{+} -ATPase were $6.78 \pm 2.30 \text{ mmol L}^{-1} \text{ red cells per hour}$.

Electron microscopic studies with the quick-freeze deep-etching method (see Section 3.2.2.2) revealed that apparent skeletal units in this disorder appeared to be normal; that is, the number of skeletal units (per μm^2) was 534 ± 35 in hereditary xerocytosis compared with 548 ± 39 in normal subjects. Electron microscopic studies with the freeze fracture method indicated that the number of intramembrane particles (IMPs) appeared to be only slightly reduced in this disorder; that is, the number of IMPs (per μm^2) was 4352 ± 215 in hereditary xerocytosis, compared with 5390 ± 420 in 20 normal individuals. In addition, the size distribution of the IMPs in this disorder demonstrated that oligomerization of band 3 molecules appeared to be slightly increased because of increased medium-sized IMPs ($42 \pm 5 \%$; normal: $27 \pm 3 \%$) concomitant with decreased normal small-sized IMPs ($49 \pm 6 \%$; normal: $71 \pm 8 \%$) [41].

A new clinical observation has been reported with recurrent fetal loss, hydrops fetalis, and familial pseudohyperkalemia (FP) [42–47]. These patients exhibit asymptomatic hyperkalemia due to an altered passive leak of potassium through the red cell membrane. In several kindred, this has been associated with xerocytosis, hydrops fetalis, and pseudohyperkalemia. The chromosomal localization for the FP turned out to be at the same location as xerocytosis [47].

Most of patients with hereditary xerocytosis appear not to require any treatment when carefully monitored for complications of increased hemolysis.

However, splenectomy may have to be considered, if the disorder becomes exacerbated [48]. The effects of splenectomy have been variable. Several patients have

developed hypercoagulability after splenectomy, and were subjected to life-threatening episodes of serious thrombo-embolism [38]. It should be noted that all cases of thrombosis have occurred after splenectomy. Increased endothelial adherence was demonstrated in the patients' red cells obtained after splenectomy [49].

The serious complication of pulmonary hypertension with an embolism was observed in four out of our eight patients with hereditary xerocytosis [41]. Therefore, these patients should be monitored under extensive medicare for these complications.

12.4

Rh_{null} Disease

The Rh (D) antigen and the other antigens of the Rh group (cCeE) are part of two minor red cell membrane proteins (see Sections 5.3.2 and 15.3.1). Patients who lack all Rh antigens (Rh_{null}) have a moderately severe hemolytic anemia with a ⁵¹Cr-labeled red cell half-life of from 10 to 14 days, stomatocytosis and occasional spherocytosis on the peripheral blood film. The Rh antigens may be significantly reduced (Rh_{mod}) in some patients [26].

The Rh antigens exist in from about 20 000 to 30 000 copies per red cell and are present on minor transmembrane proteins with an electrophoretic mobility of 28–33 kDa on SDS–PAGE gels. Rh polypeptides (c, D, and E) are distinct but closely related. Two closely linked genes exist, that is, one encoding the D polypeptide and the other encoding the Cc, Ee proteins [50–59]. The antigenic expression of these polypeptides is a consequence of alternate splicing of their pre-mRNA. The Rh proteins span the lipid bilayer several times as a membrane domain, and the C-terminus and N-terminus are located at outer and inner surfaces, respectively. The Rh proteins form dimers or oligomers, which are linked to the red cell membrane skeleton, and are substantially palmitoylated.

Although the genetic basis of the Rh deficiency syndrome is heterogeneous, at least two groups are known. The first one is the amorph type, which is related to defects involving the RH 30 locus encoding the RhD and RhE polypeptides. The second one is the regulatory type of Rh_{null} and Rh_{mod} phenotypes, which result from suppressor or modifier mutations independent of the RH 30 locus. As the pathogenesis of Rh_{null} phenotype, abnormalities of RH 50 have recently been reported, which include deletion, abnormal splicing, and missense mutations of the Rh 50 glycoprotein gene, because both the Rh locus and Rh 50 are required for the expression and function of Rh_{null} antigens as an oligomeric complex in the red cell membranes [50–59].

In Rh_{null} red cells, two proteins (32 and 34 kDa) containing extracellular thiol groups are missing. Osmotic fragility is increased in red cells of some Rh_{null} patients, indicating dehydrated red cells due to decreased cell cation and water content and increased cell density. Splenectomy appears to be effective in the reported cases. A weakened expression of Ss and U antigens has also been described, because glycophorin B is reduced by 30 % in Rh_{null} red cells.

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13

Acanthocytosis and Its Related Disorders

13.1

Introduction

The nomenclature of acanthocytes comes from the Greek *acantha*, which means “thorn”. Acanthocytes are red cells with prominent thorn-like surface protrusions that vary in width, length, and surface distribution [1–4] (Fig. 13.1). Acanthocytes are also known as spur cells, which should be distinguished from echinocytes and keratocytes. Echinocytes (Greek *echinos*: “urchin”) or burr cells are characterized by multiple small projections that are uniformly distributed over the cell surface. Keratocytes (“horn” red cells) have a few massive protrusions.

Echinocytes are a type of poikilocytic red cells, which are induced by metabolic modification of normal discocytes. The factors involved in these modifications are: (1) washing red cells with physiological saline, (2) attachment of red cells to the glass surface, (3) high alkaline pH, (4) decreased concentration of adenosine triphosphate (ATP) as the energy source for red cells, (5) accumulation of intracellular calcium in red cells, (6) the metabolic action by amphipathic compounds, and many others (see Sections 2.2.6, 2.3.4.1, and 12.1).

Normal red cells change their cell shape from discocytes through disco-echinocytes, echinocytes, and echino-spherocytes finally to spherocytes by the echinocytic pathway (Fig. 2.4). The depletion of ATP facilitates this process from normal discocytes to spherocytes, but this process is also reversible by restoration of the ATP level in red cells [5–7].

The following chemical compounds or drugs are known as echinocytogenic, that is: oleic acid, dihydroxybenzene, salicylate, 2,4-dinitrophenol, lysolecithin, ethanol,

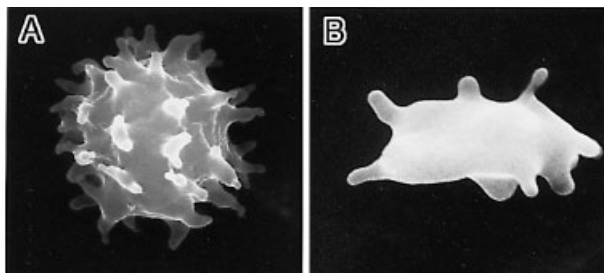


Figure 13.1 Scanning electron micrographs of echinocyte (A) and acanthocyte (B).

cholic acid, furosemide, barbiturates, tannic acid, depyridamole, lipoidal nitroxide, 1-anilino-8-naphthalene sulfonate and others. The extent of echinocytic transformation *in vitro* varies, that is: 43 % by Ca^{2+} (29 mM for 4 h), 36 % by barbiturates (83 mM for 1½ h), 87 % by salicylate (25 mM for 1 h), 42 % by saponin (0.20 mg mL⁻¹, for 1 h), and 68 % by ethanol (4.8 % for 1 h), compared with 3 % in normal control red cells (for 4 h).

This echinocytic transformation is observed in several disease states *in vivo*, such as in severe uremia, deficiencies of glycolytic enzymes (especially pyruvate kinase), conditions after splenectomy, microangiopathic hemolytic anemia, immature infants, stored blood, and many others.

Echinocytosis is also observed in association with acanthocytosis *in vivo*, such as severe hepatic dysfunction, β -lipoprotein deficiency, infantile pyknocytosis, anorexia nervosa, hypothyroidism, myelodysplastic syndrome, McLeod syndrome, rare blood group antigens such as In (Lu), and others [1–3].

Echinocytes should critically be differentiated from acanthocytes, because their are essential pathogeneses differences between them. When acanthocytes are not easily distinguished from echinocytes on a peripheral blood dry smear, the best way to achieve this differentiation is to examine the red cell shape on the unstained wet film under a light microscope with the dark field apparatus or on preparations fixed by 1 % glutaraldehyde under a scanning electron microscope (Fig. 13.1).

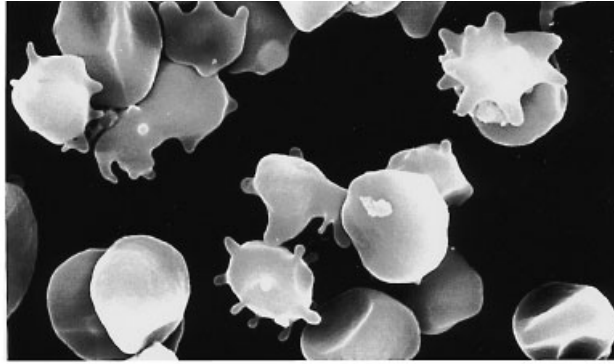
Acanthocytosis was initially described in abetalipoproteinemia, and subsequently in severe liver disease (especially, spur cell anemia), the chorea-acanthocytosis syndrome, the McLeod blood group phenotype, and other conditions [1–3]. Smaller numbers of acanthocytes (less than 10 %) may be seen in patients with myelodysplastic syndrome, hypothyroidism, anorexia nervosa, and malnutrition [1–3]. The molecular mechanisms of the formation of acanthocytes are chiefly attributed to the changes in composition of membrane lipids and their altered distribution between the outer and inner leaflets of the membrane lipid bilayer (see Sections 2. 2 and 17.6).

13.2

Abetalipoproteinemia

Abetalipoproteinemia was first described by Bassen and Kornzweig in 1950 [8]. This disorder with autosomal recessive inheritance is characterized by progressive ataxia, celiac disease, retinitis pigmentosa, and acanthocytosis [8, 9] (Fig. 13.2, see Section 17.3). The pathogenesis of this disorder lies in the deficiency of the β -lipoproteins in plasma. In this disorder, all lipoproteins that contain apoprotein B are missing in the plasma [9]. Therefore, preformed triglycerides are not transported from the intestinal mucosa, and plasma triglycerides are virtually absent. Plasma cholesterol and phospholipid levels are also significantly reduced, with a relative increase of sphingomyelin at the expense of lecithin (Table 17.5). In this mechanism, microsomal transfer protein (MTP) is crucial for catalyzing the transport of triglyceride, cholesterol ester, and phospholipid from phospholipid surfaces [9]. This MTP is a heterodimer composed of protein disulfide isomerase and a large

Figure 13.2 Scanning electron micrograph of acanthocytosis due to congenital deficiency of β -lipoproteins.



88 kDa subunit, and is located in the lumen of hepatic microsomes and intestinal epithelia, the site of lipoprotein synthesis. Thus, this microsomal transfer protein is definitely required for secretion of apoprotein B-containing lipoproteins.

Mutations of genes for apo-B100 proteins and apo-B48 proteins were initially investigated, but no mutations were observed [9]. Instead, many mutations have been detected on the gene of MTP [10–13]. Thus, the primary molecular defect in this disorder is a failure to synthesize or secrete lipoproteins containing products of the apoprotein B gene which is normally present. The defect is due to a lack of microsomal transfer protein (MTP), which catalyzes the transport of triglyceride, cholesterol ester, and phospholipid from phospholipid surfaces [9].

In this disorder, no morphological abnormalities are observed in erythroid cells from erythroblasts to reticulocytes during erythroid maturation in the bone marrow [9]. However, acanthocytosis becomes evident as the red cells mature during circulation, and worsens with increasing red cell age [9]. It is interesting to note that normal red cells, which are incubated *in vitro* with serum obtained from the patients with abetalipoproteinemia, does not produce acanthocytes, although normal red cells, which are transfused into a patient with abetalipoproteinemia, acquire acanthocytic abnormalities *in vivo*.

The degree of increased hemolysis is usually mild and anemia is basically well compensated for. Acanthocytosis is prominent (approximately 50–90% of the total red cells in peripheral blood) [8].

Plasma lipids in this disorder exhibit a striking reduction in the total cholesterol (approximately 10% of the normal level), with decreased free cholesterol and esterified cholesterol (Table 17.5). Total phospholipid content in plasma is also decreased to a level of approximately 30% that of the normal content (Table 17.5). The composition of subfractions of phospholipids indicates significant reduction of phosphatidylcholine concomitant with relatively increased sphingomyelin leading to a marked decrement in the ratio of phosphatidylcholine/sphingomyelin. In this disorder, excess sphingomyelin is preferentially confined to the outer membrane lipid bilayer leaflet, causing an expansion of its surface area that may be responsible for the irregularities in cell surface contour.

Although the decrement of plasma lipids is extremely marked, the red cell membrane lipids are surprisingly maintained at almost normal, except for an approximately 20% reduction of the phosphatidylcholine content (Table 17.5). Red cell membrane proteins are normal. No splenomegaly is observed.

Detailed descriptions on abetalipoproteinemia are available in Section 17.3.

As for other β -lipoprotein deficiencies, familial hypo- β -lipoproteinemia and chylomicron retention disease are also known. Both disorders exhibit acanthocytosis [9].

Familial hypo- β -lipoproteinemia exhibits an autosomal recessive inheritance. Homozygotes of this disorder are similar to those of abetalipoproteinemia (the Basen–Kornzweig syndrome) in clinical phenotypes with acanthocytosis, neuromuscular symptoms, lipid malabsorption and others. Through molecular genetic analyses, more than 23 types of truncated forms of the apo B-100 gene have been identified, such as mutations on apo B-2, B-9, B-25, B-27.6, B-29, B-31, B-32, B-32.5, B-37, B-39, B-40, B-46, B-49.6, B-52.8, B-54.8, B-61, B-67, B-74.7, B-82, B-86, B-87, B-89, etc. In the mutation on apo B-37, deletions at codon 5391–5394 are detected.

Chylomicron retention disease is transmitted autosomal recessively. The major clinical features are marked retention of lipid droplets in the intestinal epitheli, lipid malabsorption syndrome, acanthocytosis, neurological symptoms to a lesser extent, and others. Total low-density lipoproteins (LDL) is diminished to 50% of the normal level. The biosynthesis appears to be intact because apo B-100 is present in LDL, and apo B-48 usually exists in intestinal cells. It has been suggested that the pathogenesis exists at the secretion of chylomicron step. A possible abnormality of the glycosylated chain of apo-lipoproteins has been proposed.

13.3

Chorea-Acanthocytosis

Acanthocytosis is also observed, even under conditions with normal lipids [1–3]. A typical example is chorea-acanthocytosis syndrome [14, 15]. This disorder is transmitted autosomal recessively, and is characterized by acanthocytosis with a normal lipid content in the plasma and red cells (normolipoproteinemic acanthocytosis) and a progressive neurodegenerative disease, which exhibits (1) progressive orofacial dyskineses with tics, limb chorea, lip and tongue biting, (2) neurogenic muscle hypotonia and atrophy, (3) absence or decrease of reflexes, and (4) increased serum creatinine phosphokinase. The sites of neurological abnormalities lie on the putamen and the head of the caudate [16]. The chromosomal location of this disorder has recently been identified at the 6-cM region of 9q21 [17, 18].

Hematologically, anemia is not present, or if it may exist, it is mild. Apparent red cell survival is only slightly shortened. The acanthocytosis may be developed prior to the onset of neurological symptoms. The mechanism of acanthocytosis is unknown in this chorea-acanthocytosis. Plasma and red cell membrane lipids are essentially normal. Increased proteolysis of ankyrin, band 3, and protein 4.2 and increased membrane protein phosphorylation (especially on band 3) have been re-

ported. The missense mutation (Pro868Leu) has also been detected on the band 3 gene and has been identified in one unusual kindred with this disorder [19].

Types of congenital neuroacanthocytosis other than chorea-acanthocytosis are also known, such as: (1) one that is recessively inherited with acanthocytosis, tics, Parkinsonism, and occasional motor neuron disease; (2) a mitochondrial myopathy with acanthocytosis, encephalopathy, lactic acidosis, and stroke-like symptoms, (3) Hallervorden–Spatz disease with acanthocytosis, progressive dementia, dystonia, spasticity, and pallidal and retinal degeneration; and (4) HARP syndrome, that is hypoprebetalipoproteinemia, acanthocytosis, retinitis pigmentosa, and pallidal degeneration with iron deposition.

13.4

McLeod Syndrome

The McLeod syndrome is an X-linked anomaly of the Kell system as one of the human blood group antigens [20–22] (see Sections 5.3.5 and 15.3.2). This disorder is characterized by a mild compensated hemolytic anemia, acanthocytosis to a varying degree, and late-onset myopathy or chorea. The Kell system is composed of two protein components, that is: (1) a 37 kDa protein carrying the Kx antigen, which is a precursor molecule necessary for the Kell antigen expression, and (2) a 93 kDa protein carrying the Kell blood group antigen [22].

Patients with the McLeod syndrome exhibit no detectable Kx antigen in their red cells, and a marked deficiency of the 93 kDa protein carrying the Kell antigen [22]. Mutations of the XK gene have been identified in patients with this McLeod syndrome [23–25]. The XK gene encodes a 444 amino acid integral membrane transporter [22]. Male hemizygotes lacking the Kx antigen demonstrate 8–85 % of acanthocytes in their peripheral red cells with mild compensated hemolysis.

It is interesting to note that the red cells of the patients with a total deficiency of the Kell antigen (the Kell null: K0 red cells) show normal discocytes rather than acanthocytosis. Therefore, the McLeod syndrome should be critically distinguishable from Kell null (K0) red cells [22], in which only the 93 kDa glycoprotein carrying the Kell antigen is missing. These K0 red cells have twice the amount of the Kx antigen, which is carried in a 37 kDa protein [26].

It is known that McLeod syndrome has been reported in association with chronic granulomatous disease (CGD) in childhood, retinitis pigmentosa, and muscular dystrophy of a Duchenne type [27]. These combined phenotypes may come from contiguous gene syndromes, in which the gene loci of these two disorders are located on Xp21 sufficiently close to each other. Some patients with McLeod syndrome may develop myopathy and late-onset neurological manifestations, such as areflexia, progressive dystonia and choreiform movements.

Amongst the many blood group antigens, other than the Kell blood group antigen, patients with the *In (Lu)* Lu (a–b–) phenotype may develop acanthocytes and poikilocytes [22, 28], although anemia has not been reported. *In (Lu)* is a dominantly acting inhibitor, and suppresses expression of Lu^a and Lu^b, which are two

major antigens of the Lutheran blood group system (see Section 5.3.4). Their abnormal red cells of the *In* (*Lu*) *Lu* (a–b–) phenotype become osmotically resistant and lose potassium during the incubation stage of the osmotic fragility test [29].

13.5

Spur Cells and target Cells

Spur cells, or acanthocytes, are present in the blood films of patients with severe hepatic dysfunction [1–3] (see Sections 2.2.6 and 17.6). Abnormal red cell membrane lipid composition and altered lipid distribution between the inner and outer leaflets of the membrane lipid bilayer characterize this clinical phenotype.

The most serious form of hepatic dysfunction is spur cell anemia [30] (Fig. 13.3). As the prevalence of liver disease is so high these subjects account for the majority of patients with acanthocytosis observed in routine clinical medicine, although only a small number of patients at the final stage of hepatic dysfunction develop this serious spur cell anemia.

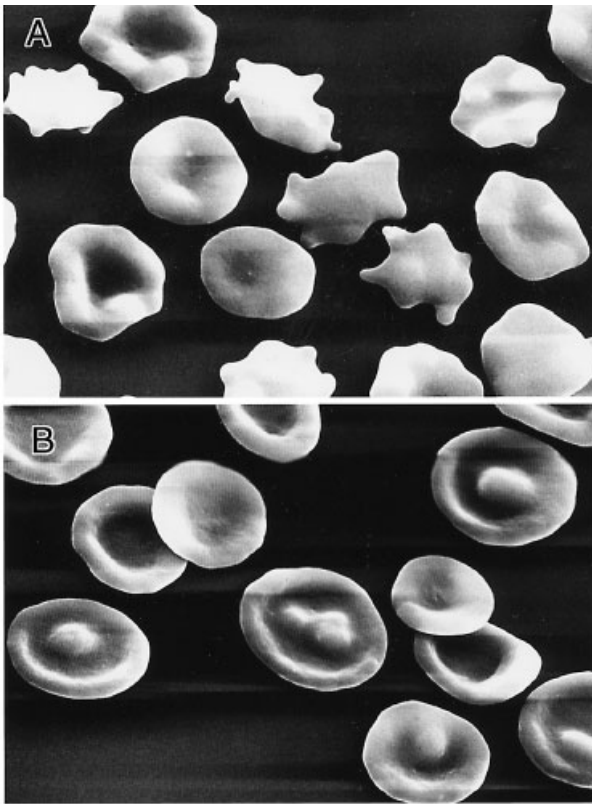


Figure 13.3 Scanning electron micrographs of spur cells (A) and target cells (B).

The formation of a spur cell *in vivo* is initiated by the accumulation of nonesterified free cholesterol in the patient's red cell membrane and remodeling of the red cells with abnormal morphology by the spleen [31, 32]. The acquisition of free cholesterol from plasma into the patient's red cells is enhanced by abnormally high cholesterol/lipoprotein ratios [31]. Through this mechanism, free cholesterol is readily incorporated into the red cell membrane and is preferentially distributed in the outer leaflet of the membrane lipid bilayer. Increased free cholesterol is known as a potent hardening factor for membrane lipid fluidity, and makes the red cell membrane less fluid. The spleen tends to remodel the membrane leading to rigid, spherical red cells with the spiculated projections characteristic of spur cells [32]. These red cells with decreased deformability appear to be trapped in the narrow sinusoids of the splenic circulation and are auto-hemolyzed.

Clinically, spur cell anemia is characterized by a rapidly progressive hemolytic anemia with acanthocytosis in peripheral blood [30]. Splenomegaly and jaundice are striking in association with extensive ascites, bleeding diatheses due to coagulopathy and thrombocytopenia, and hepatic encephalopathy. The incidence of spur cell anemia is high in patients with alcoholic liver disease, but also with advanced metastatic liver malignancies, hepatic cirrhosis, Wilson disease, fulminant hepatitis, and other disease conditions. The patients exhibit moderate to severe anemia with a hematocrit of 20–30 %, a marked elevation of the indirect bilirubin level, and always the presence of severe hepatic dysfunction. The presence of spur cell anemia is a serious indicator of the terminal stages of severe liver disease. Prognosis for the patients at this stage with spur cell anemia is serious, probably only a few weeks of life.

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14

Abnormalities of Skeletal Proteins

14.1

α -Spectrin

14.1.1

Introduction

Red cell spectrin (α I β I Σ 1 spectrin) is the principal red cell skeletal protein [1–5], and contains two large polypeptide chains that are structurally similar but functionally distinct, that is, α -spectrin (280 kDa) and β -spectrin (246 kDa) (see Sections 2.3, 3.2.2, 4.1, 10.4 and 11.3). The molecular weight by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) is 240 kDa in α -spectrin and 220 kDa in β -spectrin. The two chains are aligned side by side in an antiparallel arrangement with respect to their N- and C-terminal ends. Each spectrin chain is composed of a series of 106 amino acid repeats connected end to end. The primary sequence of each repeat shows extensive symmetry with conserved hydrophobic residues which are located in the first and fourth positions of each seven residue motif. Each segment folds into three helices (helix A, B, and C; or helix 1, 2, and 3) connected by short nonhelical portions. The helices fold back against each other to form triple helical bundles, which are stabilized by interaction of hydrophobic side chains of conserved residues in each helix. Helices in adjacent repeat segments also interact with each other.

α -Spectrin (see Sections 4.1.1–4.1.3) starts with an isolated, unpaired C helix followed by nine characteristic spectrin repeats (No. 1–9), a tenth repeat of an src homology (SH3) domain (No. 10), and a further 12 more spectrin repeats (Nos. 11–22). The tenth segment of the SH3 domain appears to be an attachment site for a family of tyrosine kinase binding proteins. The C-terminal region of α -spectrin contains two EF hands, which is a characteristic for the regulation of calcium action in other proteins such as α -actinin and fordrin which is an α II β II spectrin.

β -Spectrin (see Sections 4.1.1–4.1.3) is composed of 17 conformational repeats (Nos. 1–17). The fifteenth and the proximal section of the sixteenth repeats are modified and are the binding site for ankyrin. The N-terminal region of 272 residues contains the binding sites for actin and protein 4.1. This part is homologous to other actin-binding proteins (α -actinin, dystrophin, adducin, etc.). The C-terminal region of red cell β -spectrin is composed of a 52 amino acid extension with four

phosphorylation sites (3P-Ser, and 1P-Thr) for a membrane-associated casein kinase I. Membrane mechanical stability can be decreased by increased phosphorylation.

Tryptic digestion of the spectrin peptides exhibits a series of proteolytically resistant domains joined by protease sensitive regions. Nine such domains are known, that is, five on the α -spectrin (α I through α V), and four on the β -spectrin (β I through β IV). These spectrin domain maps, after proteolytic digestion by trypsin, are of critical importance and are useful for identification of molecular abnormalities of α - and β -spectrins in hereditary elliptocytosis (see Section 11.3.2).

From the viewpoint of spectrin functions, spectrin oligomerization is also crucial (Fig. 4.3, see Section 4.1.3). The α - and β -spectrins assemble to form a heterodimer through a side-to-side interaction. The formation of a pair of these two spectrins occurs in a zipper-like fashion, which starts with a defined nucleation site composed of four repeats from each spectrin chain at the end opposite the self-association site, repeats α 19 to α 22, and β 1 to β 4. Two of the four α repeats and one of the four β repeats have an eight residue insertion in the 106 amino acid repeat unit. An α -spectrin allele, α^{LELY} , which is a Low Expression allele LYon, produces α chains that lack one of the nucleation sites and do not form stable heterodimers [6] (see Sections 11.3.3 and 11.4). This allele is clinically important.

Human beings have at least five spectrin genes, four of which are related to the plasma membrane (see Section 4.1.4). (1) α I Spectrin is only detected in mature red cells. The α I spectrin gene (*SPTA*) is located on chromosome 1q22–q23 near the Duffy blood group. (2) α II Spectrin is found in almost all cells except for mature red cells. The α II spectrin gene (*SPTAN 1*) is located on chromosome 9q34.1. (3) β I Spectrin is present in red cells (the S1 isoform), muscle and brain (the S2 isoform). The chromosomal location of the β I spectrin gene (*SPTB*) is on 14q23–q24.2. (4) β II spectrin is widely distributed, as is the α II spectrin. The β II spectrin gene (*SPTBN 1*) is on chromosome 2p21. (5) β III spectrin is found on membranes of Golgi apparatus and intracellular vesicles, but not on plasma membranes. The β III spectrin gene (*SPTBN 2*) is on chromosome 11q13. Thus, spectrin in mature red cells (spectrin_R) contains the α I and β I S1 chains. Muscle spectrin is α II β IS2. Most other tissues are composed of α II β II, which in other nomenclature systems is known as fodrin, tissue spectrin, brain spectrin, or spectrin_G.

During erythroid development and maturation (see Chapter 7), it is now known that red cell membrane proteins are expressed sequentially. Spectrin is first synthesized very early probably in immature erythroid-committed stem cells such as colony forming units-erythroid (CFU-E), and burst forming units-erythroid (BFU-E), and is abundant in proerythroblasts. Glycophorins and band 3 follow after the spectrins have been expressed. Ankyrin and protein 4.1 appear at the middle stage of erythroid differentiation, and protein 4.2 is finally expressed at the very late stage of erythroid maturation, around polychromatic and orthochromatic erythroblasts. During this process, α -spectrin is expressed before β -spectrin is expressed. In addition, α -spectrin is synthesized in at least a three-fold excess relative to β -spectrin, and is degraded by a different slower pathway. In contrast, β -spectrin exhibits a limited synthesis and more rapid degradation. Thus, β -spectrin is definitely rate-limiting in the spectrin assembly. This difference in the rate of produc-

tion of α - and β -spectrin is critical to the understanding of the molecular pathogenesis of red cell membrane disorders as regards the abnormalities of spectrins (see Chapter 11). The best example is that α -spectrin anomalies are not observed in autosomal dominantly inherited hereditary spherocytosis, in spite of the fact that a substantial number of β -spectrin abnormalities are reported in this disorder, probably because defective α -spectrin synthesis in one allele is not expected to cause a disease state, and also β -spectrin synthesis is rate-limiting.

14.1.2

α -Spectrin Abnormalities

In most cases of hereditary elliptocytosis and hereditary pyropoikilocytosis (see Sections 11.3 and 11.4), there is a defect in the oligomerization of spectrin. The ability of spectrin to form oligomers can be determined by utilizing spectrin which is extracted from the membrane at 0 °C (see Sections 4.1.2 and 4.1.3). At this low temperature, the formation and dissociation of spectrin tetramers and oligomers are greatly reduced. Under these conditions, the proportion of each spectrin species (dimers, tetramers, and oligomers) indicates its relative proportion on the membrane *in situ*. Approximately 30% of patients with hereditary elliptocytosis and all patients with hereditary pyropoikilocytosis in Western countries have a defect in their ability to assemble spectrin dimers into tetramers and higher-order oligomers. This observation indicates that the underlying defects of these patients may lie in the spectrin heterodimer self-association site. In fact, many structural abnormalities in either α - or β -spectrin have been found in this region by analysis of isolated spectrin peptides from these patients with hereditary elliptocytosis.

Spectrin peptide structural analyses (see Sections 4.1.2 and 4.1.3) can be performed by two-dimensional isoelectric focusing–SDS–polyacrylamide gel electrophoresis, after spectrin from these patients has been partially digested by trypsin at 0 °C. With this procedure, five trypsin-resistant domains of the α -spectrin (α I– α V), and four domains of the β -spectrin (β I– β IV) can be identified. In patients with hereditary elliptocytosis (see Section 11.3.2), abnormal peptide fragments appear at various positions when compared with the normal due to the generation of new, abnormal tryptic cleavage sites in the mutant spectrin chains, such as a mutated $\alpha^{I/74}$ instead of normal $\alpha^{I/80}$. Many mutations of spectrins, especially of α -spectrin, have now been identified in patients with hereditary elliptocytosis by the procedures described above. The majority of the α -spectrin variants are characterized by a decrease or absence of the normal 80 kDa α I domain with the appearance of a smaller proteolytic fragment from the mutated α -spectrin. Thus, most of the α -spectrin mutations lie in the α I domain at the N-terminal region that forms part of the spectrin self-association site (Fig. 14.1). The naming of these mutations is based on the nomenclature by Antonarakis [7]. In most of these mutations of α -spectrin, the mutations alter the two-dimensional tryptic maps and lie close to the site of the abnormal tryptic cleavage site. Most of them are located in helix C (or helix 3) of the spectrin repeats and appear to disrupt the triple helical structure of the repeats.

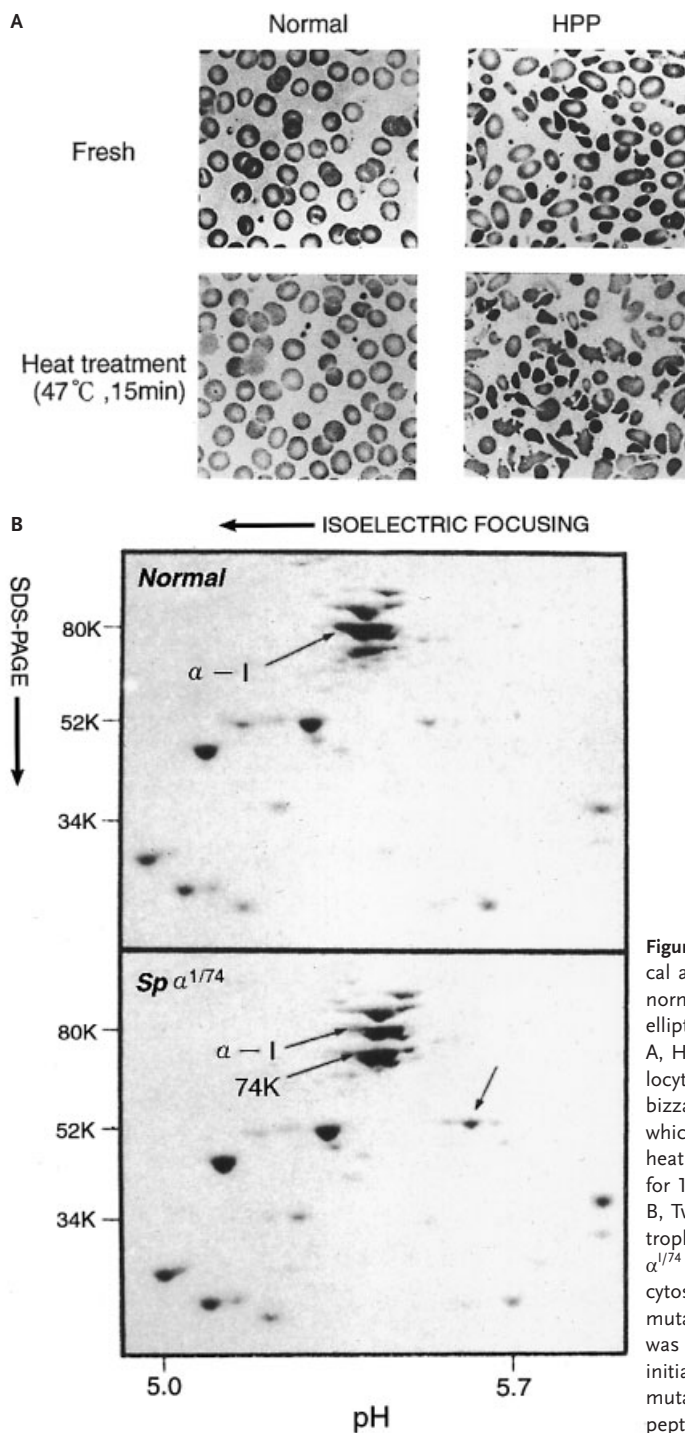


Figure 14.1 Morphological and molecular abnormalities of hereditary elliptocytosis.

A, Hereditary pyropoikilocytosis (HPP) with bizarre red cell shape which is enhanced by heat treatment at 47°C for 15 min.

B, Two dimensional electrophoresis of spectrin $\alpha^{1/74}$ in hereditary elliptocytosis. The presence of mutated 74 kDa peptide was observed which was initially derived from the mutation of $\alpha^{1/80}$ kDa peptide of α -spectrin.

The reported cases (Table 11.1) (see Section 11.3.3) are: (1) α -spectrin Lograno (I 24S), (2) α -spectrin Corbeil (R28H), (3) anonymous (R28C), (4) anonymous (R28L), (5) anonymous (R28S), (6) α -spectrin Marseille (V31A), (7) α -spectrin Genova (R34W), (8) α -spectrin Tunis (R41W), (9) α -spectrin Clichy (R45S), (10) α -spectrin Anastasia (R45T), (11) α -spectrin Culoz (G46V), (12) anonymous (K48R), (13) α -spectrin Lyon (L49F), (14) α -spectrin Ponte de Sôr (G151D), (15) anonymous (L154–155 insertion at codon 154), (16) α -spectrin Dayton (E178–E226 deletion at codon 178), (17) α -spectrin Saint Louis (L207P), (18) α -spectrin Nigerian (L260P), (19) anonymous (S261P), (20) α -spectrin Sfax (1086 A→G at codon 363), (21) α -spectrin Alexandria (H469 deletion at codon 469), (22) α -spectrin Barcelona (H469P), (23) anonymous (Q471P), (24) α -spectrin Jendouba (D791E), (25) α -spectrin Oran (2465–1G→A at codon 822), and (26) α -spectrin St. Claude (2806–13T→G at codon 936) (Table 11.1).

In these above mentioned mutations (see Section 11.3.3), the $\alpha^{I/74}$ defect is heterogeneous and is caused by mutations in codons 24, 28, 31, 34, 46, 48 or 49. There are four mutations found at codon 28, which contains a CpG dinucleotide, suggesting that this codon appears to be a hot spot of gene mutations. Regarding the clinical severity, the mutations in codons 41, 46 or 49 are associated with milder forms, whereas those in codon 45 or 48 are associated with severe phenotype such as hereditary pyropoikilocytosis. The $\alpha^{I/65}$ defect exhibits the duplication of a leucine residue in codon 154 although the phenotype is mild, the same as the mutation in codon 151. The $\alpha^{I/50}$ defect is associated with substitution of a proline which is known to disrupt α helix formation, a 49 residue deletion in codons 178 to 226 (α -spectrin Dayton), or a single residue deletion in codon 469 (α -spectrin Alexandria). The $\alpha^{I/36}$ mutation is due to deletion of codons 363 to 371 due to activation of a cryptic splice site (spectrin Sfax). The $\alpha^{II/31}$ mutation is caused by a point mutation (D791E), and the $\alpha^{II/21}$ defect is due to skipping of exon 18 by a deletion of codons 822 to 862, from a mutation in the acceptor splice site for exon 18 (spectrin Oran). In α -spectrin St. Claude, a splice site mutation causes premature termination of the α -spectrin, abolishing the spectrin dimer nucleation site. The mutated α -spectrin molecules appear to lack the spectrin dimer nucleation site located near the C-terminus and not to be incorporated into the membrane skeleton. The homozygous proband with spectrin St. Claude demonstrates severe hemolytic anemia, in contrast to its heterozygotes which are asymptomatic with normal red cell morphology, probably because α -spectrin synthesis is normally approximately three-fold greater than β -spectrin, and enough α -spectrin is produced by the single normal α -spectrin allele to bind all of the β -spectrin and form a complete membrane skeleton (see Chapter 7).

It is extremely interesting to note that only very few truncated α -spectrins are reported in patients with hereditary elliptocytosis. Most of gene mutations of α -spectrin are missense mutations which are clustered at the N-terminal region of the α -spectrin molecule, whereas most of the β -spectrin mutations are abnormal skipings of exons, frameshift mutations, or nonsense mutations, which are more strictly limited to the C-terminal region of the β -spectrin molecule.

Generally speaking, in α -spectrin mutations, the abnormal cleavage sites that produce the mutated peptides are not the primary defects but are due to conformational changes of the spectrin chains associated with these defects.

Among these abnormalities of α -spectrin, there is an inverse relationship between the distance of the defect from the spectrin dimer self-association site, (such as the N-terminal region of α -spectrin) and its associated clinical severity. The closer to the self-association site, the greater the functional defect, and the more severe the clinical phenotype. The best example is the $\alpha^{I/74}$ defects, which are present right at the spectrin dimer self-association site, exhibit the most severe clinical phenotype such as life-threatening hemolysis in homozygotes, and a greater proportion of spectrin dimers (15–40%) even in heterozygotes. Instead, in the $\alpha^{I/65}$ defects, which are located much further away from the self-association site of the N-terminus of α -spectrin, milder hemolysis and a lesser defect in spectrin self-association are observed. Furthermore, in the $\alpha^{II/21}$ defect, which is located at a distance a long way from the self-association site, the disease is very mild even in the homozygote.

It is known that there are some patients with severe hemolytic anemia, who are diagnosed as having a typical common type of hereditary elliptocytosis. These patients carry the α -spectrin mutation on their one allele. The extent of the hemolysis should usually be mild, appearing as common hereditary elliptocytosis. However, the clinical phenotype in these patients is severe, disproportionately more than what could be expected. Thus, these patients inherit a second, low-expression allele, which is silent in carriers but contributes to the severity of the disease when present *in trans* to a structurally mutated allele. This idea, proposed by Delaunay et al. in Lyon, was proven by the discovering of the fact that a common polymorphism ($\alpha^{V/41}$) is associated with the reduced incorporation of α -spectrin into the membrane skeleton. The low expression allele of α -spectrin is known as α^{LELY} (Low Expression LYon) [6], after the peptide that is reduced in quantity [6] (see Section 11.3.3).

The α^{LELY} allele is linked to two abnormalities, that is, (1) a missense mutation (L1867V in exon 40) causing an abnormal tryptic cleavage after the amino acid at codon 1920 at the $\alpha^{\text{IV-V}}$ junction, resulting in the variant peptide ($\alpha^{V/41}$), and (2) a C→T substitution in an acceptor splice site 12 nucleotides before the splice junction that leads to 50% in-frame skipping of exon 46. This exon is 18 base pairs long, and lies within the nucleation site for α -spectrin to β -spectrin association, although this exon is too small to be detected by SDS–polyacrylamide gel electrophoresis. As the nucleation site of α -spectrin is interfered with, the α -spectrin lacks α^{LELY} in exon 46 and fails to bind to β -spectrin or to be assembled into the membrane skeleton, or else they are destroyed. The remaining α -spectrin, which contain the exon 46, exhibits a normal function.

The α^{LELY} allele *per se* is completely silent, even when homozygous, because the extent of α -spectrin synthesis is in a three-fold higher excess than is required, and the α^{LELY} splicing mutation causes only partial skipping of exon 46. However, when the α^{LELY} allele occurs *in trans* to a second allele which carries a structurally abnormal α -spectrin pathognomonic for hereditary elliptocytosis, then the mutant α -

spectrin with the structural defect will be preferentially incorporated into the membrane. This definitely aggravates the clinical severity of hereditary elliptocytosis. When a mutation of hereditary elliptocytosis is present *in cis* on a chromosome carrying the α^{LELY} mutation, an essentially mild defect can be converted into an asymptomatic one or a severe one into a mild clinical phenotype. In contrast, when a seriously mutated allele of hereditary elliptocytosis is present *in trans* to the α^{LELY} allele, the clinical phenotype results in a much more severe type, such as severe hereditary elliptocytosis, or hereditary pyropoikilocytosis.

The incidence of the α^{LELY} allele is 31 % in Caucasian and 20 % in Japanese populations (see Section 11.3.3). This polymorphism also prevails widely at a very high frequency in other ethnic groups, such as Africans, Taiwanese, and Amazon Indians. The variability of clinical phenotypes of hereditary elliptocytosis, which is often encountered in medical practice, can be accounted for fairly well, even if not totally.

Contrary to an enormous contribution to the pathogenesis for hereditary elliptocytosis including hereditary pyropoikilocytosis, α -spectrin abnormalities in one allele do not play an important role in pathogenesis for hereditary spherocytosis (see Chapter 10), probably because α -spectrin is normally synthesized in an approximately three-fold excess (see Chapter 7), and most of autosomal dominantly inherited hereditary spherocytosis are heterozygotes, in which only one allele could have mutated.

In some patients, however, a life-threatening form of non-dominantly inherited hereditary spherocytosis is reported in association with marked spectrin deficiency, in which the spectrin content is approximately 25–50 % of the normal level (see Section 10.4.4). In such families, variants of α -spectrin are detected as αIIa or α -spectrin Bughill. The mutated α -spectrin exhibits a substitution of A970D of the α -spectrin gene. In these patients, only the αIIa variant is detected by peptide analysis of the α -spectrin, although both an allele with the $\alpha\text{IIa}/\alpha$ -spectrin Bughill mutation and the other allele without this mutation are observed at the genomic DNA level. This result indicates that the second α -spectrin allele in these patients may be silent in its function. A candidate gene for this silent mutation is now known in a family with severe non-dominant hereditary spherocytosis. The first one is α -spectrin Prague (at nucleotide 5187–2A→G at codon 1730) [9], which has a mutation in the position second from the end of intron 36, leading to skipping of exon 37 and premature termination of the α -spectrin. The other α -spectrin allele demonstrates a partial splicing abnormality in intron 30, and produces only about one-sixth of the normal amount of α -spectrin. This low expression allele is known as α -spectrin LEPRa (Low Expression allele PRaGue: at nucleotide 4339–99C→T at codon 1449) [10], which is linked to the $\alpha\text{IIa}/\alpha$ -spectrin Bughill mutation in this patient and in several other patients with non-dominant hereditary spherocytosis. The interaction of α -spectrin LEPRa and an α -spectrin allele encoding a nonfunctional peptide appears to be fairly frequent in the pathogenesis of severe non-dominant hereditary spherocytosis [11, 12].

Animal models for hereditary spherocytosis [13, 14] are known in the common house mouse, *Mus musculus*. These anemias resemble human hereditary spherocytosis. In these mice, anemia is observed only in the homozygotes and all of the mu-

tants (*sph/sph*, *sph^{ha}/sph^{ha}*, *sph^{2BC}/sph^{2BC}*, *sph^{1J}/sph^{1J}*, *sph^{2J}/sph^{2J}*, and *sph-Dem/sph-Dem*) have severe hemolysis along with marked reticulocytosis, spherocytosis, jaundice, bilirubin gallstones, and massive hepatosplenomegaly. The mutations are autosomal recessive.

Of the mutants described above, the *sph/sph* mutants lack α -spectrin with small amounts of β -spectrin. These *sph/sph* variants have defects in the synthesis, function, and/or stability of α -spectrin [14]. The *sph* and *sph^{2BC}* alleles are frameshift mutations and null mutations [15]. The *sph^{1J}/sph^{1J}* mice synthesize normal amounts of spectrin mRNA and protein, although these mice have a nonsense mutation near the C-terminus that deletes the last 13 amino acids from the α -spectrin. These amino acids in the EF hand region at the C-terminal end of α -spectrin are expected to be important functionally in attaching spectrin to actin. In fact, the α -spectrin produced in the *sph^{1J}/sph^{1J}* mice is not stably incorporated into the membrane. The *sph-Dem/sph-Dem* mice are derived from in the CeS3/Dem strain, and exon 11 and 46 amino acids are missing from repeat 5 of the α -spectrin molecule [16]. The mice demonstrate both spherocytosis, elliptocytosis, and some poikilocytes, resembling a combined phenotype of hereditary spherocytosis and hereditary pyropoikilocytosis in humans.

14.2

β -Spectrin

14.2.1

Introduction

The α - and β -spectrin chains assemble into a heterodimer through a side-to-side interaction (see Sections 2.3, 3.2.2, 4.1, 10.4 and 11.3). The spectrin heterodimers further self-associate to form tetramers and higher oligomers by interacting in a head-to-head configuration. Tetramers are the predominant form of spectrins *in vivo*. At low ionic strength and physiologic temperature (at 37 °C), spectrin dissociates into dimers. In contrast, at physiologic ionic strength and lower temperature (at 25 °C), tetramers predominate. At 4 °C, the equilibrium is almost kinetically frozen because of its high activation energy. Therefore, it is possible to extract spectrins from the red cell membrane at these temperatures and to examine their association states in detail. It is now known that formation of spectrin tetramers and higher oligomers is essential in maintaining the mechanical strength of the membrane skeleton.

The structure of the spectrin self-association site can be elucidated by analyses of mutant spectrins and synthetic spectrin fragments, and by proteolytic studies. The isolated C helix (or helix 3) at the N-terminus of α -spectrin associates with the isolated A and B helices (or helix 1 and helix 2) at the C-terminus of β -spectrin to form a stable triple helical spectrin repeat and a head-to-head orientation of the spectrin molecules. The isolated helices alone are not sufficient for this interaction. At least one adjacent triple-helical spectrin repeat should exist. Mutations in α - or β -spectrin

with disruption of this triple helical coil interaction inhibit the formation of spectrin tetramers and higher oligomers leading to weakening of the membrane skeleton. The most common cause of hereditary elliptocytosis and hereditary pyropoikilocytosis lies in these defects (see Section 11.3.3), especially at the C-terminal end of β -spectrin.

The C-terminal region of β -spectrin is phosphorylated just beyond the site of self-association. Phosphorylation of β -spectrin appears to have no effect on spectrin self-association *in vitro*, but *in vivo* it decreases the membrane mechanical stability markedly [17, 18].

Functionally, spectrin appears to be a type of spring (see Sections 2.3 and 3.2.2.1). The tetramers are coiled up *in vivo* with an end to end distance of about 76 nm. The spectrin molecules condense by twisting their α and β subunits around a common axis, and the degree of condensation is regulated by varying the pitch and diameter of the twisted double strand. The coiled spectrin tetramers can extend reversibly until they are stretched up to 200 nm as their maximum length. These observations are confirmed by electron micrography.

14.2.2

β -Spectrin Abnormalities

In hereditary elliptocytosis, at least 19 mutations in β -spectrin have been reported (see Section 11.3.3). These mutations are all located near the C-terminal region of β -spectrin which is the spectrin dimer self-association site.

Molecular defects causing truncated β -spectrin are as follows (Table 11.1). Abnormal splicings with skipping of exon 30 are: (1) β -spectrin Le Puy (at nucleotide 6219+4 A→G), the same as our β -spectrin Yamagata; (2) β -spectrin Göttingen (at nucleotide 6219+2 T→A); (3) β -spectrin Campinas (at nucleotide 6219+1 G→A); and (4) β -spectrin Prague (at nucleotide 6023–1 G→C). Abnormal splicing with skipping of exon 31 is observed in (5) β -spectrin Rouen (at nucleotide 6269+3 G→T). In these cases with abnormal splicings at the C-terminal end of the β -spectrin gene in the patients with hereditary elliptocytosis, the overall length of mutant β -spectrin is 2008 amino acids in cases (1) through (4), and 2104 amino acids in case (5) of β -spectrin Rouen. The amounts of mutated β '-spectrin are 10–42% of the total β -spectrins. Frameshift mutations of β -spectrin are also described, that is: (1) β -spectrin Nice (at nucleotides 6136–6137 of codon 2046 in exon 30: insertion of GA), (2) β -spectrin Tandil (at nucleotides 6124–6130 of codon 2041 in exon 30: deletion of seven nucleotides), (3) β -spectrin Tokyo (at nucleotide 6177 of codon 2059 in exon 30: deletion of C), and (4) β -spectrin Napoli (at nucleotides 6160–6167 of codon 2053 in exon 30: deletion of eight nucleotides). One nonsense mutation is β -spectrin Nagoya (E2069X), and several missense mutations are (1) β -spectrin Cagliari (A2018G), (2) β -spectrin Kayes (A2053P), (3) β -spectrin Cosenza (R2064P), (4) β -spectrin Paris (A2023V), (5) β -spectrin Linguere (W2024R), (6) β -spectrin Providence (S2019P), (7) β -spectrin Cotonou (W2061R), (8) β -spectrin Buffalo (L2025R), and (9) β -spectrin Kuwaitino (A2018D). In several of these missense mutations, nucleotide substitutions to a proline residue are observed in their

mutant peptide, such as β -spectrin Kayes, β -spectrin Cosenza, and β -spectrin Providence. As proline is known to disrupt the α -helices, the substitution to proline is critical for the triple helical conformation of spectrin in maintaining its structural stability. In most of these β -spectrin mutations, an α -spectrin abnormality of $\alpha^{1/74}$, which is a variant from normal $\alpha^{1/80}$, is also observed on tryptic digestion maps of spectrins, as shown in β -spectrin Tokyo in the following section. This is now believed to be the result of the exposure of the N-terminal region of α -spectrin to increased tryptic digestion due to the abnormal spectrin self-association caused by the β -spectrin mutations.

Patients with β -spectrin mutations exhibit mild to moderate hemolysis with hemoglobin levels of 8.0–15.2 g dL⁻¹ and reticulocytosis of 1.8–30 %. Osmotic fragility in these patients is usually increased, and thermal stability is abnormal when they are examined. Homozygous patients with β -spectrin mutations demonstrate a clinical phenotype of hereditary pyropoikilocytosis with severe neonatal hemolysis or even lethal hydrops foetalis (see Section 11.2).

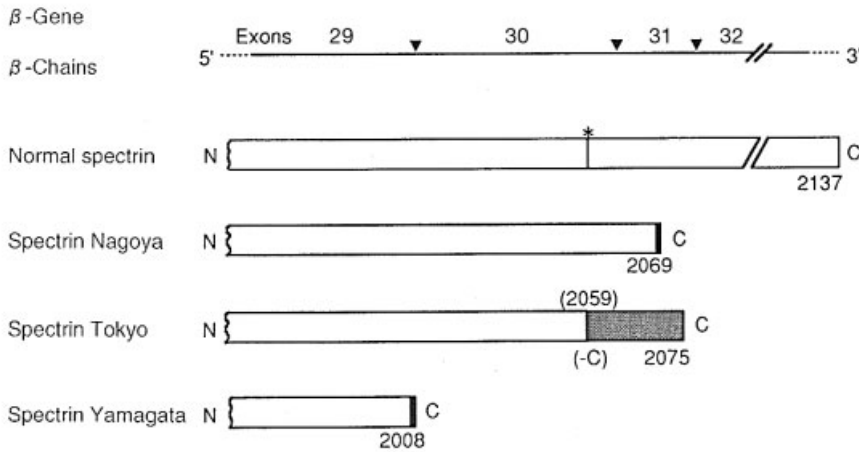
The pathogenesis of the abnormal membrane functions lies in the altered spectrin self-association by disrupting the coiled-triple helical structure that forms the spectrin dimer self-association site (see Section 11.3.2). In the cases with β -spectrin anomalies of $\beta^{220/216}$, peptides of approximately 4 kDa are lost from the C-terminal region of β -spectrin resulting in the truncation of the β -spectrin molecule of 216 kDa compared with 220 kDa of a normal β -spectrin molecule. In addition, low temperature spectrin extracts contain increased spectrin dimer up to approximately 50 % of the dimer of the total dimer–tetramer content compared with normal (5–10 %).

Table 14.1 Genotypic and phenotypic characteristics in Japanese HE patients with β -spectrin gene mutations.

	<i>Inheritance</i>	<i>Last normal position</i>	<i>Extent of $\alpha\beta$-spectrin deficiency</i>	<i>Mutation</i>	<i>Size of replacing missense sequence (amino acids)</i>	<i>Overall length of mutant β-chain</i>	<i>Mutant spectrin ($\beta'/\beta + \beta' \times 100\%$)</i>	<i>Hemoglobin (g dL⁻¹)</i>	<i>Reticulocytes (%)</i>
(Spectrin Yamagata)									
Abnormal splicing	AD	2007	Normal	nt+4a→g (intron 30); exon 30 skipped	1	2008	35	8.3	12.6
(Spectrin Tokyo)									
Frameshift mutation	AD	2059	Normal	1 nt. del. at codon 2059 in exon 30	16	2075	16	8	10
(Spectrin Nagoya)									
Nonsense mutation	<i>de novo</i>	2069	Normal	2069: GAG→TAG; Glu→stop codon	—	2069	16	8.1	9

β' : truncated β -spectrin, AD: autosomal dominant inheritance, nt: nucleotide, del: deletion.

A



B

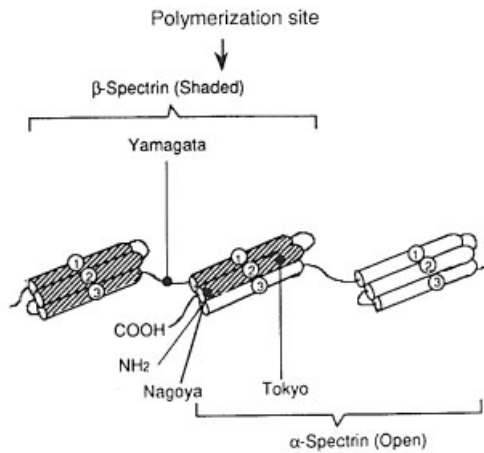


Figure 14.2 Truncated β -spectrin molecules in β -spectrins Nagoya, Tokyo, and Le Puy in Yamagata.

A: Gene structure,
B: mutation sites.

Nearly all of the abnormal truncated β -spectrin (β^{216}) is detected in the dimer fraction, indicating that the inability of the abnormal truncated β -spectrin (β^{216}) peptide to oligomerize is responsible for the critical defect of spectrin functions. Details are shown in β -spectrin Tokyo, β -spectrin Le Puy in Yamagata, and β -spectrin Nagoya in the following sections (Table 14.1 and Fig. 14.2).

Surprisingly, β -spectrin abnormalities have also recently been discovered in patients with hereditary spherocytosis (Table 10.1), although these have long been considered as the pathogeneses specific for hereditary elliptocytosis including hereditary pyropoikilocytosis. A group of patients who are heterozygous for defects in

the β -spectrin are associated with isolated spectrin deficiency and autosomal dominantly inherited hereditary spherocytosis (see Section 10.4.4). The majority of β -spectrin mutations have been associated with null alleles, including frameshift, nonsense, and initiation codon mutations. The sites of these mutations spread widely over the whole β -spectrin molecule, but mostly at the N-terminal actin–protein 4.1 binding domain and in the first half region of the spectrin repeats, but none at the C-terminal end of the β -spectrin molecule, which is characteristic of hereditary spherocytosis compared with those for hereditary elliptocytosis. At least 19 mutations are described in dominant hereditary spherocytosis and only one mutation (β -spectrin Birmingham: R1684C) is described in recessive hereditary spherocytosis. The frameshift mutations are (1) β -spectrin Ostrava (at nucleotide 604 at codon 202: deletion of T), (2) β -spectrin Bicêtre (at nucleotide 1331 at codon 444: deletion of eight nucleotides), (3) β -spectrin Philadelphia (at nucleotides 1767–1768 at codon 590: insertion of A), (4) β -spectrin St. Barbara (at nucleotide 1912 at codon 638: deletion of C), (5) β -spectrin Bergen (at nucleotides 2351–2352 at codon 785: insertion of A), (6) β -spectrin Bergen (at nucleotide 2351–2352 at codon 783–784: insertion of A), (7) β -spectrin Houston (at nucleotide 2777 at codon 926: deletion of A), and (8) β -spectrin Durham (deletion from L at codon 1492 to K at codon 1614). The nonsense mutations are (1) β -spectrin Promissão (M1V), (2) β -spectrin Alger (Q514X), (3) β -spectrin Baltimore (Q845X), and (4) β -spectrin Tabor (Q1946X). Abnormal splicing mutations are (1) β -spectrin Guemene-Penfao (at nucleotide 300 at codon 100: G→C), and (2) β -spectrin Winston-Salem (at nucleotide 3764+1 at codon 936: G→A). The missense mutations are (1) β -spectrin Atlanta (W182G), (2) anonymous (G189A), (3) β -spectrin Kissimmee (W202R), (4) β -spectrin Oakland (I220V), (5) β -spectrin Columbus (P1227S), (6) β -spectrin Birmingham (R1684C), and (7) β -spectrin Sao Paulo (A1884V).

Amongst these mutations, null alleles including frameshift, nonsense and initiation codon mutations, gene deletions and splicing defects appear to produce serious dysfunctions of β -spectrin, which is especially true in β -spectrin Durham, in which an enormous 4.6 kb genomic deletion is observed. The truncated peptides produced are incorporated inefficiently into the red cells. In β -spectrin Winston-Salem, exon skipping results in an unstable truncated β -spectrin peptide, and similarly in β -spectrin Guemene-Penfao, which causes an intron to be retained and disturbs the production of β -spectrin transcripts. A frameshift mutation of β -spectrin, due to a single nucleotide (A) deletion in β -spectrin Houston, is detected in patients from several unrelated kindreds, suggesting that this mutation might be a common β -spectrin mutation with hereditary spherocytosis, although most other mutations are private. β -Spectrin Kissimmee (W202R) is unique among several missense mutations. In this defect, heterozygous patients have two types of spectrin. The abnormal fraction, approximately 40 %, cannot bind protein 4.1 and therefore binds weakly to actin. The defect is located at the N-terminal region of the β -spectrin, where the actin–protein 4.1 binding site exists. The substitution of Trp (W) to Arg (R) in β -spectrin Kissimmee at codon 202 inserts a positively charged amino acid in a highly conserved region of a largely hydrophobic amino acid sequence and could disrupt a region which is critical for binding to protein

4.1. The mutated β -spectrin is unstable and susceptible to thiol oxidation. These abnormalities appear to induce the defect into its binding to protein 4.1. Since it is known that patients with the spectrin–protein 4.1 binding abnormality demonstrate reduced (80 % of the normal content) spectrin content, it is feasible to speculate that the patient with β -spectrin Kissimmee exhibits spherocytosis. In any case, the mutated β -spectrin, which is produced by these various types of gene mutations, detaches from the membrane more easily than normal and is subjected to increased proteolysis.

In animal models, six types of hereditary hemolytic anemia have been identified in the common house mouse, *Mus musculus* [13, 14]. These anemias resemble hereditary spherocytosis in human beings. Among them, the *ja/ja* mutant exhibits no detectable spectrin. The mice carry a nonsense mutation in the β -spectrin gene and lack the ability to produce stable β -spectrin. The pathogenesis lies in replacement of an arginine with a stop codon in the mRNA encoding the ninth repeat of β -spectrin. In *ja/ja* mice in adulthood, cardiac thrombi, fibrotic lesions, and renal hemochromatosis are observed [18, 19]. The membrane vesicles, which are released from the extremely unstable mouse red cells, appear to expose phosphatidylserine on their outer surface, which would be very thrombogenic. The same line of discussion has been made in clinical hematology; that is, severe and fatal portal vein thrombosis is one of the major hazardous complications in patients with paroxysmal nocturnal hemoglobinuria with severe hemolysis.

14.2.3

β -Spectrin in Tokyo

The representative β -spectrin variant is β -spectrin Tokyo ($\beta^{220/216}$) with HE [20]. It carries a truncated β -chain (β' -chain) and displays a severely impaired dimer self-association. The β' -chain could not be phosphorylated *in vitro*. Genomic and cDNA sequencing showed a 1-base deletion in codon 2059 belonging to exon 30 of the β -spectrin gene. As a result, a missense sequence extended down to (new) codon 2075. Serine 2060, a potential phosphorylation site, was replaced by alanine.

The proband (Y. I.) is an 8 month old girl with severe neonatal jaundice and moderate uncompensated hemolysis. RBC analysis showed elliptocytosis (nearly 90 %). The patient's mother presented with slightly increased hemolysis and typical elliptocytosis identical to that of the proband.

An abnormal band (β') was detected between normal β -spectrin and ankyrin upon SDS–PAGE of membrane proteins in the proband and her mother (Fig. 14.3). This band had an apparent molecular weight of 216 kDa and was identified as spectrin by immunoblotting with antispectrin polyclonal antibodies. Using an anti- β -spectrin monoclonal antibody, it was further ascertained that it was β -spectrin. The β' -chain could not be phosphorylated *in vitro*.

The amounts of normal spectrin with respect to protein 4.2 was slightly reduced in the proband and her mother, but not in the father. The β/α ratio was also diminished in the proband and her mother, but the $(\beta + \beta')/\alpha$ ratio was normal. Critically, the proportion of the β' -chain was low (15.8 % in the proband and 17.4 % in her mother).

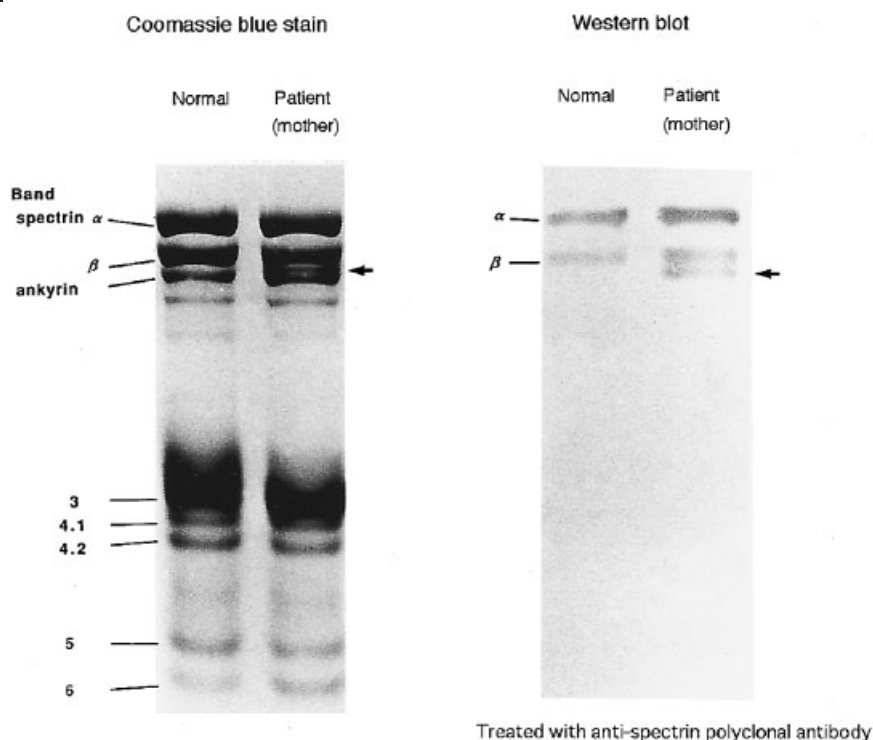


Figure 14.3 Detection of truncated β' -spectrin by SDS-PAGE and Western blotting with anti-spectrin antibody in a patient of β -spectrin Tokyo. Mutated β' -spectrin is shown by an arrow.

The percentage dimer in crude spectrin extracts (4 °C) was increased in the proband and her mother, 27.4 and 29.0%, respectively, compared with 11.9% in the father and $10.6 \pm 3.4\%$ as the normal ($n = 12$). Dimer to tetramer conversion showed an increased amount of spectrin remaining as a dimer in the proband and her mother. The association constant, K_a ($\times 10^5 \text{ L mol}^{-1}$), was 3.6 and 3.2 in the proband and her mother, respectively, versus 6.2 in her father and 5.5 ± 1.4 ($n = 68$) in the control. The defect in dimer self-association was further supported by the distribution of the β' -chain. In the crude spectrin extract (4 °C) of the proband, it appeared essentially in the dimers and additionally in the high molecular weight (HMW) oligomers, not in the tetramers. The presence of the β' -chain in the HMW oligomers is probably accounted for by the persistence, in this fraction, of some actin and protein 4.1 that hold together the junctional complex, including some $\alpha\beta'$ dimers. Finally, the β' -chain remained in the dimers upon dimer to tetramer conversion. Thus, the $\alpha\beta'$ dimer is unable to participate in tetramerization.

As seen in other spectrin variants with a shortened β -chain, the proband and her mother showed a reduction of the αI 80 kDa fragment and a reciprocal increase of the αI 74 kDa fragment upon partial trypsin digestion.

cDNA and genomic DNA sequencing indicated the deletion of one C in codon 2059 producing a frameshift with a missense sequence downstream, although amino acid 2059 (alanine) remained unchanged (Table 11.1. II). Regarding potential phosphorylation sites, serine 2057 was preserved, whereas serine 2060 was replaced. The missense sequence was interrupted by a TGA codon, which normally overlaps codons 2076 and 2077 (CTGAAA).

Spectrin Tokyo ($\beta^{220/216}$) is a variant with a truncated β -chain. As in similar mutants, the change causes the $\alpha\beta'$ dimer to be virtually unable to tetramerize. It also induces a conformational change in the facing α -chain that results in an accelerated production of the αI 74 kDa fragment upon partial trypsin digestion. All these features bring further support to the model developed by Tse et al., according to which helix 2 of repeat $\beta 17$, which is profoundly modified in spectrin Tokyo as well as in other variants with a truncated β -chain, interacts with helix 3 of repeat $\alpha 1$ so as to create the dimer self-association site.

Serine 2057 and 2060 are potential phosphorylation sites in the spectrin β -chain. The truncated β' -chain of spectrin Rouen, which has a normal sequence down to position 2073, has retained some phosphorylation capacity. The shortened β' -chain of spectrin Nice, which has a normal sequence down to position 2045 and, of course, other abnormal β' -chains with truncation beginning further upstream, have lost their phosphorylation ability. In spectrin Tokyo, which can no longer be phosphorylated, serine $\beta 2057$ is conserved, whereas serine $\beta 2060$ is replaced. This suggests that serine 2060 is a possible phosphorylation site in normal spectrin.

Known variants with a truncated β -chain are clinically manifest in the heterozygous state (their expression level is sometimes low; see below), while the only variant with a point mutation reported so far is clinically asymptomatic. However, *in vitro* tetramerization was disturbed in the latter case. In addition to the interaction between helix 2 of repeat $\beta 17$ and helix 3 of repeat $\alpha 1$ mentioned above, the 52 amino acid unique sequence after repeat $\beta 17$ (C-terminal region of the β -chain) may play a critical role in stabilizing the tetramer. Alternatively, a few amino acids eliminated by whatever deletion has been described so far may be critical for self-association. On the other hand, tetramerization was barely altered in a spectrin mutant, the β -chain of which is thought to carry an 84 kDa C-terminal extension.

In terms of overall length, spectrin Tokyo (2075 amino acids) is nearly identical to spectrin Nice (2076 amino acids). Indeed, both variants depend on the same stop codon. The one amino acid difference (three nucleotides) is accounted for by the summation of the deletion (one nucleotide) and the insertion (two nucleotides) that characterize both abnormal β -alleles, respectively. In spectrin Tokyo, the missense sequence (16 amino acids) is shorter than in spectrin Nice (31 amino acids). It is puzzling, then, that spectrin Tokyo has a lower incorporation level than spectrin Nice. At the posttranscriptional stage, the mutation does not apparently activate a cryptic splice site that would cause part of the pre-mRNA to be abnormally spliced and eventually degraded. At the posttranslational stage, a smaller missense sequence would have been expected to produce a smaller instability and greater in-

corporation into the membrane, spectrin Tandil, resulting from yet another frame-shift mutation, which also has a low incorporation level. Amongst the spectrin variants in which the shortened β -chain arises from exon skipping, only spectrin Rouen presents with an incorporation level as low as spectrin Tokyo.

One may wonder why the presence of 10 to 15 % of a truncated β -chain in the membrane, e.g., approximately one of six β -spectrin molecules, results in a clinically manifest picture. A 10 to 15 % level of an ($\alpha^{1/74}$) α -chain would not produce clinical symptoms as far as we know at present. We may postulate that ($\alpha^{1/74}$) β -mutations leading to truncation of the β -chain disturb more profoundly the self-association process. This, again, would stress the role of the last 52 amino acids of the spectrin β -chain (in addition to the role of helix 2 belonging to repeat $\beta 17$). Tetramers with one truncated β -chain would be entirely unable to assemble, as *in vitro* studies tend to corroborate, whereas those with one mutated α -chain would form to some extent and thus appear as partly functional. From studies by Liu et al., we would expect that spectrin Tokyo substantially disrupts the skeletal lattice even in the heterozygous state.

14.2.4

β -Spectrin Le Puy in Yamagata

The second example of β -spectrin mutation is β -spectrin Le Puy ($\beta^{220/214}$) in Yamagata [21]. The propositus (60 years old) is a Japanese patient with moderate jaundice (bilirubin: total 3.72 and indirect 2.48 mg dL⁻¹) and uncompensated anemia. The red cell morphology of the peripheral blood from the daughter of the propositus with the spectrin Le Puy mutation demonstrates a marked elliptocytosis.

The patient's mother was deceased due to cerebrovascular disease and his sister died of a severe hemolytic anemia at the age of 30. His brother is healthy and does not have signs of hemolysis. His 36 year old daughter has a hemolytic anemia with typical elliptocytosis.

Protein structure and function An abnormal band (β' ; apparent MW: 214 kDa) was detected between normal β -spectrin and ankyrin on the SDS-PAGE of membrane proteins in the propositus. It was ascertained as being a spectrin β -chain using immunoblotting with polyclonal antibodies to spectrin α - and β -chains and, specifically, with a monoclonal antibody to the spectrin β -chain. The percentage of the β' -chain [$\beta'/(\beta + \beta')$] was 31.0 %. The β' -chain could not be phosphorylated *in vitro* using ghosts and radio-labeled ATP. The dimer percent [$\text{SpD}/(\text{SpT} + \text{SpD})$] in crude spectrin extracts (4 °C) from the propositus and his daughter was increased and the association constant, K_a , was decreased (3.2 and 2.9) (Fig. 14.4). In the crude spectrin extract (4 °C) of the propositus, the β' -spectrin appeared to a great extent in the spectrin dimers [$\beta'/(\beta + \beta')$], (77.2 %) and slightly in the tetramers (8.8 %). These data stressed an impaired ability of the $\alpha\beta'$ dimer to participate in tetramerization. Upon partial trypsin digestion of spectrin for 22 h, there was an increase of the $\alpha 1$ 74 kDa fragment at the reciprocal expense of the $\alpha 1$ 80 kDa fragment (33.80 %; normal 25.62 ± 0.42). This indicated that the β' -chain in-

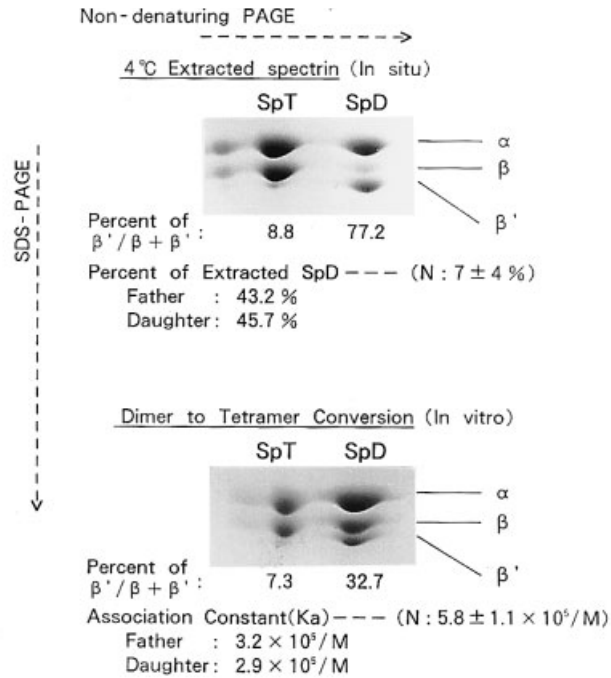


Figure 14.4 Presence of the β' -chain essentially in spectrin dimers due to impaired self-association of truncated β -spectrin anomaly in hereditary elliptocytosis (β -spectrin Yamagata). Oligomers, tetramers (SpT), and dimers (SpD) of spectrins were separated horizontally using nondenaturing PAGE. The chain content of each fraction was resolved vertically using SDS-PAGE. The β' -chain was essentially present in spectrin dimers. These findings *in situ* were also confirmed by those *in vitro*.

duced a change in the α -chain within the self-association site. Altogether, all features characterizing ($\alpha^{1/74}$) β -mutants were present.

In the proband, nucleotide sequencing showed that the 343 bp segment lacks exon 30, and revealed an A→G substitution at position +4 of the 5' donor splice site consensus sequence of intron 30 (Table 11.1. II). Genomic DNA analysis of the proband's daughter disclosed the same abnormality.

The membrane skeletons were examined under native unstretched conditions by QFDE electron microscopy. In normal subjects, the ghosts exhibited a web-like appearance with short rods and small globules accounting for spectrin intersections (e.g., junctional complexes). The alignment of the network appeared orderly. In contrast, the skeleton appeared disrupted in the proband, with filaments of uneven lengths and widths, and a reduction in the number of spectrin intersections.

The membrane skeletons in spectrin Le Puy in Yamagata were also examined by EM with the surface replica method. The basic cytoskeletal units varied in size, and were mostly enlarged. All of the fibrous filaments appeared to lose their interconnections with other filaments. It is interesting to note that some of the fibrous

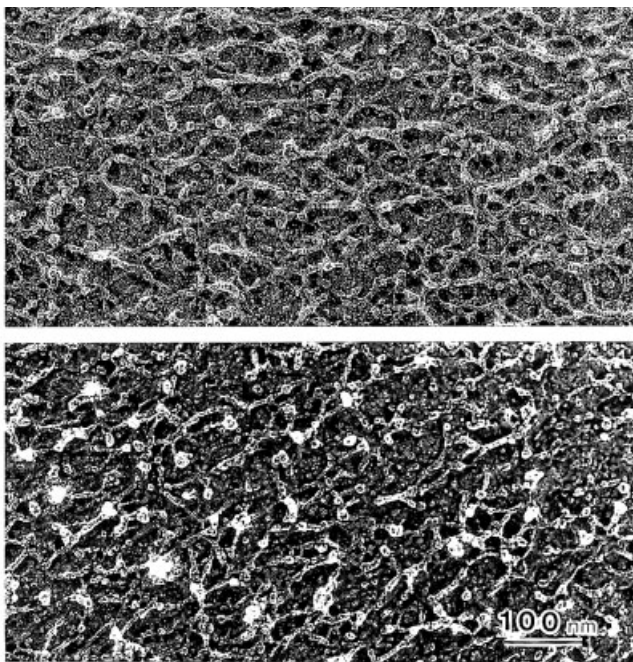


Figure 14.5 Deranged cytoskeletal network in β -spectrin Le Puy in Yamagata examined by electron microscopy with the quick-freeze deep-etching method. A continuous orderly three-dimensional network of fine filaments and small globules is observed in a normal subject (the upper part). In contrast, the structure of the cytoskeletal network in a patient with β -spectrin Le Puy in Yamagata appears disrupted with filaments of uneven lengths and widths and a reduction of the number of intersections (the lower part).

filaments had a drum-stick appearance, which probably reflected the irregularly coiled condition of these filaments. The structure of the whole red cell membranes in this disorder was clearly disorganized. Each fibrous filament were identified as being spectrins by the immunogold method.

In spectrin Le Puy in Yamagata, the continuous three-dimensional network of fine filaments and small globules which had been observed in the normal control was totally disrupted with filaments of uneven lengths and width. A reduction in the number of intersections was observed by the EM with the QFDE method (Fig. 14.5). The alignment of the network appeared disorderly. These findings were essentially the same as those by EM with the surface replica method. It seems difficult for abnormal β -spectrin to maintain a normal tight cytoskeletal network. As a result, the units may easily become uncoiled.

Since erythroid β -spectrin is also expressed in muscle and in the eye lens, a careful examination of the neuromuscular system and of the eye was performed at the clinical level, and revealed no specific signs.

14.2.5

 β -Spectrin Nagoya

The third example is β -spectrin Nagoya [22]. A new HE β -spectrin shortened variant, designated β -spectrin Nagoya is described. The truncation stemmed from a primary stop codon (E2069X) in exon 30.

Scanning electron microscopy of a male baby as the proband red cells disclosed a pronounced elliptocytosis. Protein analysis of proband's red cell membrane on SDS-PAGE gave evidence of an additional band of 217 kDa (apparent molecular weight), accounting for 16.3% of the total β -spectrin (normal β -chain: 84.7%) (Table 14.1). The 217 kDa band reacted with anti-spectrin polyclonal antibodies and was not phosphorylated *in vitro*.

A T-to-G substitution at the first nucleotide of codon 2069 (GAG→TAG; Glu→stop), in exon 30, was observed after proband cDNA sequencing, and designated E2069X (a mutation also found at the gene level by sequencing) (Table 11.1. II). The new stop codon generated a loss of the 69 C-terminal amino acid residues in the variant β -spectrin chain, yielding a variant termed β -spectrin Nagoya. The presence of the mutation was at the heterozygous state, and suggested that the mutation was *de novo*.

Mutations in β -spectrin associated with HE, an impaired self-association process and a β -chain truncated in its C-terminal region, have already been described. Most of the primary mutations leading to a frameshift are clustered in exon 30 or in the corresponding exon-intron boundaries. β -Spectrin Nagoya is an additional member of the present family but is the first variant in which the truncation stems from a primary stop codon (E2069X).

14.2.6

Correlation Between Genotype and Phenotype Expressions in β -Spectrin Anomalies

There has been recent progress in the field of red cell membrane disorders using molecular biological techniques especially with regard to spectrin anomalies. Compared with numerous reported cases of α -spectrin mutations, β -spectrin anomalies are relatively rare, and only 20 variants have been reported, three of which were identified at the Kawasaki Medical School [20–21]. On these mutations of β -spectrin in human red cells, it was determined that there were varying degrees of truncation of the C-terminal region of β -spectrin based on molecular abnormalities. In contrast to β -spectrin Nagoya [22] which has the least truncation, β -spectrin Le Puy [21] demonstrated the most marked truncation of the β -spectrins. β -Spectrin Tokyo [20], β -spectrin Nice, β -spectrin Tandil, β -spectrin Kuwaitino, β -spectrin Cagliari, β -spectrin Providence and others are in between. It is known that the C-terminal region of β -spectrin is bound to the N-terminal region of α -spectrin ($\alpha^{1/80}$). The head-to-head contact of α - and β -spectrins initiates the formation of the hetero-tetramer ($\alpha_2\beta_2$). Therefore, truncation at the C-terminal region of β -spectrin is expected to induce impairment of the head-to-head association between α - and β -spectrins. Morphologically, the impairment of the association should be indicative of the dis-

ruption of the cytoskeletal network; that is, a disconnection of the fibrous filaments with uneven lengths and widths, and a reduction in the number of intersections. Among the various β -spectrin mutants described above, the red cell membrane skeletal network would be most impaired in Spectrin Le Puy, because the mutation of the β -spectrin gene in β -spectrin Le Puy is known to induce maximal truncation of the C-terminal region of β -spectrin.

Phenotypic expressions were evaluated in three β -spectrin (Sp) anomalies, that is, β -Sp Le Puy, β -Sp Tokyo and β -Sp Nagoya, which were with frameshift and nonsense mutations.

Considering the genotypic characteristics, β -Sp Le Puy [21] had a point mutation (A $\rightarrow$ G) at the +4 nucleotide of the 5'-donor splice site of intron 30. β -Sp Tokyo [20] showed the deletion of C at codon 2059. β -Sp Nagoya [22] was due to a nonsense mutation of E2069X. All of these three mutations skipped the exon 30. The last normal codon was 2007 in β -Sp Le Puy, 2059 in β -Sp Tokyo, and 2068 in β -Sp Nagoya, compared with the normal subjects (2137). Therefore, the extent of truncation of the C-terminal region of β -Sp was -130 amino acids in β -Sp Le Puy, -78 in β -Sp Tokyo, and -69 in β -Sp Nagoya.

As to the phenotype characteristics, in clinical hematology, red cell counts in the peripheral blood were $2.62 \times 10^{12} \mu\text{L}^{-1}$ in β -Sp Le Puy, 3.42 in β -Sp Tokyo, and 3.92 in β -Sp Nagoya. Elliptocytosis was most marked with many rod-shaped elliptocytes in β -Sp Le Puy, moderately ovalocytic in β -Sp Tokyo, and minimally ovalocytic in β -Sp Nagoya (Fig. 14.6). Biochemically, the mutated β^1 -Sp content was 36 % of total Sp content in β -Sp Le Puy, 17 % in β -Sp Tokyo, and 16 % in β -Sp Nagoya. The content of Sp dimer and the self-association constants of spectrin dimers were 43 % and $3.2 \times 10^5 \text{ M}^{-1}$ in β -Sp Le Puy, 28 % and 3.6 in β -Sp Tokyo, and 22 % and 4.0 in β -Sp Nagoya, respectively (Normal: 7.0 ± 4.0 and 5.8 ± 1.1). Therefore, phenotypically, β -Sp Le Puy appeared to be most severely affected, β -Sp Tokyo was moderate, and β -Sp Nagoya was minimal. Electron microscopically, the states of the skeletal network in these red cells *in situ* were evaluated by the quick-freeze deep-etching method (Fig. 14.7) (see Section 3.2.2.2). The membrane ultrastructure was most disrupted in β -Sp Le Puy, moderate in β -Sp Tokyo, and minimal in β -Sp Nagoya. The number of basic skeletal units was significantly reduced ($334 \pm 30 \mu\text{m}^{-2}$) in β -Sp Le Puy, 410 ± 38 in β -Sp Tokyo, and 480 ± 50 in β -Sp Nagoya (normal: $548 \pm 30 \mu\text{m}^{-2}$). Intramembrane particles (IMPs) were also examined by EM with the freeze fracture method (see Section 3.2.3). The number of IMPs was $3800 \pm 208 \mu\text{m}^{-2}$ in β -Sp Le Puy, 3915 ± 305 in β -Sp Tokyo, and 4828 ± 370 in β -Sp Nagoya (normal: 5390 ± 420). Reflecting on the abnormal skeletal network, IMPs were considerably clustered and unevenly distributed in β -Sp Le Puy, less in β -Sp Tokyo, and least in β -Sp Nagoya. The extent of genotypic abnormalities, which determines the length of normal amino acid chains of the β -Sp molecule in these β -Sp anomalies, was well correlated with the phenotypic expressions (Fig. 14.8), such as the extent of anemia in clinical hematology, biochemical abnormalities of red cell spectrin, and the abnormalities of membrane ultrastructure *in situ*.

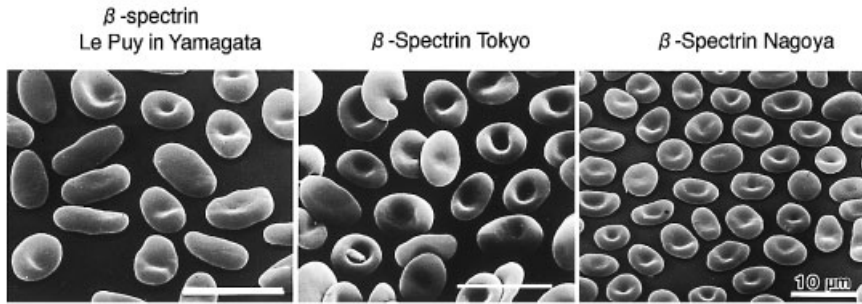


Figure 14.6 Scanning electron micrographs of peripheral red cells in β -spectrins Le Puy in Yamagata, Tokyo, and Nagoya.

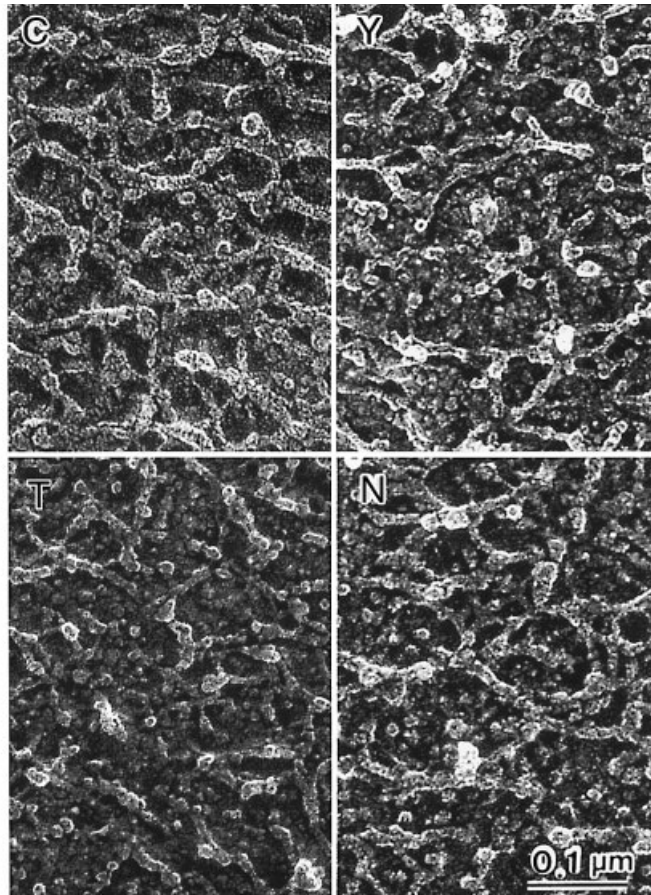


Figure 14.7
Electron micrographs of red cell cytoskeletal networks in a normal control subject (C), and β -spectrins Le Puy in Yamagata (Y), Tokyo (T), and Nagoya (N) examined by the quick-freeze deep-etching method.

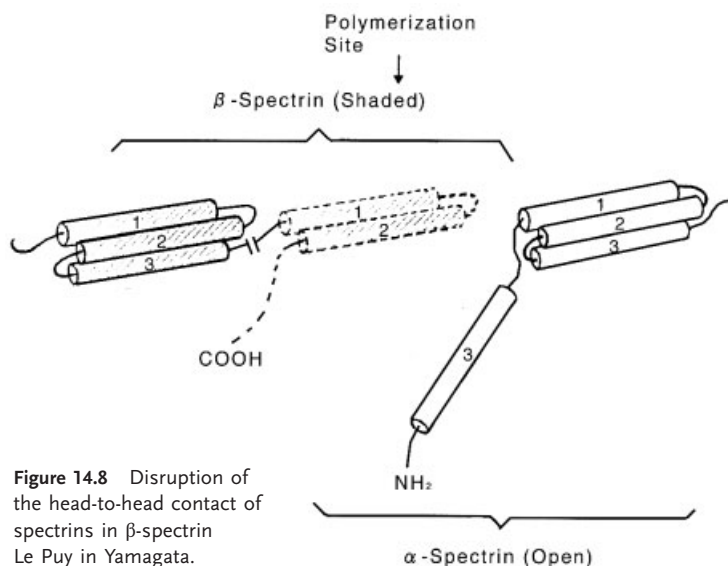


Figure 14.8 Disruption of the head-to-head contact of spectrins in β -spectrin
Le Puy in Yamagata.

14.3 Protein 4.1

14.3.1 Introduction

Protein 4.1, which is one of the key proteins of the skeletal network, has been studied extensively and has been shown to be an intriguing multifunctional structural protein using biochemistry and molecular biology (see Sections 2.3, 4.2 and 11.3). It is present in approximately 200 000 copies per cell and interacts with the skeletal proteins, spectrin and actin, to form the junctional complex. It also associates with integral proteins, glycophorin C, and band 3. *In vitro* proteolysis studies have shown protein 4.1 to be composed of four major domains of 30, 16, 10, and 22/24 kDa. It participates in interactions with spectrin and actin at its 10 kDa domain and is also connected to transmembrane proteins by its 30 kDa domain. Therefore, protein 4.1 appears to play an important role in the horizontal associations of skeletal proteins, but also anchors the skeletal network to transmembrane proteins vertically. Despite such detailed information, which has been obtained mostly by *in vitro* experiments, the exact molecular arrangement of membrane proteins (especially protein 4.1) and their interactions with the intact membrane structure *in situ* have not been determined.

Clinically, various abnormalities in protein 4.1 have been reported, including structural mutations of protein 4.1 and quantitative abnormalities such as 4.1 (–) hereditary elliptocytosis (HE); i.e., a heterozygote with reduced protein 4.1 or a homozygote with no protein 4.1 (see Chapter 11).

The red cell protein 4.1 (protein 4.1R) gene (*EPB41*) is located on chromosome 1p36.1 (see Section 4.2).

The protein 4.1 gene is large (>200 kb) and complex with at least 23 exons, including 13 alternatively spliced exons. This results in a markedly diverse variety of protein 4.1 isoforms in erythroid and nonerythroid cells, each containing different peptide cassettes (motifs).

An upstream 17 base pair sequence at the 5' end of exon 2 contains an in-frame ATG codon that is included in protein 4.1R isoforms which are observed in most nonerythroid cells (see Sections 4.2.3 and 4.2.4). The resulting protein contains an additional 209 amino acids at the N-terminus and has an apparent molecular weight of 135 kDa by SDS–polyacrylamide gel electrophoresis. This sequence is omitted early in erythroid differentiation, and a downstream ATG is utilized instead, resulting in the typical 80 kDa isoform of protein 4.1 which is present in mature red cells. Total deficiencies of protein 4.1R have been observed in four independent kindred (protein 4.1 Algeria, protein 4.1 Annery, protein 4.1 Lille, and protein 4.1 Madrid), in which the downstream initiation codon of the protein 4.1R gene is affected, as shown below. Three other mutations have been reported (Table 11.1. III).

Alternative splicing of exon 16 is triggered late in erythroid differentiation and inserts a 21 amino acid sequence into the 10 kDa domain that is essential for its binding to spectrin and actin. In the pathological conditions, two qualitative variants (protein 4.1^{68/65}, and protein 4.1⁹⁵) are known when considering the 10 kDa spectrin–actin binding domain of protein 4.1 (see Section 11.3.3).

This remarkably diverse alternative splicing of protein 4.1 makes the analyses of the protein 4.1 gene extremely difficult, although a large number of patients with partial protein 4.1 deficiency exist.

Protein 4.1 in mature red cells is composed of four structural domains (see Section 4.2.1), which are defined by proteolysis, (1) 30 kDa, (2) 16 kDa, (3) 10 kDa, and (4) 22/24 kDa from the N-terminus to the C-terminus. The 30 kDa domain includes binding sites for calmodulin, CD44, glycophorin C, band 3, and protein p55. The 10 kDa domain contains binding sites for myosin, spectrin, and actin. The 22/24 kDa domain contains two posttranslational modifications. The first one is gradual and spontaneous amidation of asparagine at codon 502 of the protein 4.1R gene, which converts protein 4.1b with an apparent molecular weight of 78 kDa into protein 4.1a of 80 kDa. The process is red cell age-dependent. The second one is an O-linked monosaccharide, N-acetylglucosamine, which is found on cytoplasmic protein 4.1.

Protein 4.1 has many sites binding it to other membrane proteins on its molecule (see Section 4.2.2). First of all, protein 4.1 can bind to spectrin and actin. The binding site of protein 4.1 to β -spectrin is present very near the actin-binding site. The spectrin–actin binding sites are present at the 10 kDa domain of protein 4.1, which is composed of a 21 amino acid spliced alternatively with exon 16 and constitutive exon 17. The 21 amino acid sequence and residues 37 to 43 in the constitutive exon 17 form the spectrin binding site alongside an actin-binding site, which is located at the beginning of the constitutive sequence. In addition, one

molecule of protein 4.1 can bind to one monomer of F-actin, resulting in conformational changes in the actin filament that promotes further protein 4.1 binding. Thus, protein 4.1 appears to connect spectrin to actin. The spectrin–actin binding by protein 4.1 is blocked by protein kinase A phosphorylation in tyrosine at codon 418 in the 10 kDa domain of the protein 4.1 molecule. The ternary complex also appears to be regulated by Ca^{2+} and calmodulin. Calmodulin binds to protein 4.1 in a Ca^{2+} -independent manner. However, the calmodulin–protein 4.1 complex appears to infer a Ca^{2+} -sensitivity on its binding to spectrin and actin.

When protein 4.1 is depleted, in the patients with total deficiency of protein 4.1 (such as protein 4.1 Madrid), the red cells exhibit marked hemolysis and membrane instability. The reconstitution of protein 4.1 or its fragment of 64 amino acids, which are the 21 amino acid spectrin–actin binding sites in exon 16 and the next 43 amino acids in exon 17, restores normal membrane stability.

Secondly, protein 4.1 also associates with glycoporphins, especially the cytoplasmic regions of both glycoporphin A and glycoporphins C and D *in vitro*. Of these, the interaction of protein 4.1 with glycoporphins C and D is the functionally much more important than with glycoporphin A. Glycoporphins C-deficient red cells exhibit elliptocytosis with membrane instability, but glycoporphin A-deficient red cells show normal discocytes with normal stability of the membrane. In addition, the cytoplasmic domain of glycoporphins C and D contains two sites for protein 4.1. Protein 4.1 binds directly to the proximal site near the lipid bilayer, and p55 binds to the distal site near the C-terminus of glycoporphins C and D. Protein 4.1 binds to a positively charged portion in the middle of p55.

Thirdly, protein 4.1 also binds to band 3. Regarding the binding sites, there is one site near the N-terminus of band 3 and two sites containing positively charged motifs (LRRRY and IRRRY) near the start of the transmembrane domain of band 3. Approximately 20% or less of the protein 4.1 appears to be bound to band 3 on the membrane. The interaction is blocked by phosphorylation of protein 4.1 by protein kinase C.

Finally, protein 4.1R interacts with many other proteins and phospholipids, such as myosin, tubulin, the neuronal membrane protein paranodin, the immunophilin FKBP13, the volume-regulated chloride channel pICln, CD44, the nuclear mitotic apparatus protein NuMA, three tight junction proteins (ZO-1, ZO-2, and occludin), and phospholipids, especially phosphatidylserine and polyphosphoinositides. These negatively charged phospholipids are located mainly in the inner leaflet of the membrane lipid bilayer. Protein 4.1 may have a regulatory function with respect to its membrane binding due to its low-affinity.

There are four protein 4.1 genes including the red cell protein 4.1 gene (*EPB41*) (see Section 4.2.4). The protein 4.1N gene (*EPB41L1*) is a homologue that is strongly expressed in the nervous system. The protein 4.1G gene (*EPB41L2*) is a general form that is widely expressed. The protein 4.1B gene (*EPB41L3*) is another brain specific analogue. The protein 4.1 itself is part of a superfamily of ERM proteins that includes ezrin, radixin, moesin, merlin, talin, coracle and several tyrosine phosphatases. They share their homology with the 30 kDa N-terminal domain.

14.3.2

Protein 4.1 Abnormalities

In some patients with hereditary elliptocytosis protein 4.1 is deficient partially or totally (see Chapter 11). In heterozygotes, protein 4.1 is decreased by around 50 % in the common type of hereditary elliptocytosis with prominent elliptocytosis, no red cell fragmentation, and virtually no increased hemolysis. Partial deficiency of protein 4.1 is a major cause of hereditary elliptocytosis in Japan. In contrast, in homozygotes, protein 4.1 is absent in a severe hemolytic anemia with markedly increased osmotic fragility, bizarre red cell morphology, and effective splenectomy. Extremely fragile red cells with total deficiency of protein 4.1 in homozygotes can restore membrane stability by reconstituting with purified protein 4.1 or a recombinant protein 4.1 fragment of the spectrin–actin binding region.

With a total deficiency of protein 4.1, four independent mutations are known: (1) protein 4.1 Algeria, (2) protein 4.1 Annery, (3) protein 4.1 Lille, and (4) protein 4.1 Madrid (see Section 11.3.3). Of these, protein 4.1 Algeria is caused by a 318 base pair deletion in the protein 4.1R gene, which eliminates the downstream initiation codon utilized by the 80 kDa erythroid isoform of protein 4.1. In the same way, protein Annery is also caused by a large 70 kb deletion of the protein 4.1R gene with elimination of the downstream initiation codon. Protein 4.1 Lille (M1T) and protein 4.1 Madrid (M1R) have missense mutations in the same initiation codon, which also prevent expression of the erythroid type of protein 4.1. Patients with complete protein 4.1 deficiency also demonstrate a virtual deficiency of glycophorin C, because protein 4.1 has a binding site to glycophorin C in the membrane. A detailed description on protein 4.1 Madrid will be given in the following section.

Deletion of a single residue in the 10 kDa spectrin–binding domain of the protein 4.1 gene is observed in protein 4.1 Aravis (K 447 deletion), resulting the loss of its capacity to bind spectrin. Two other mutations have also been described (Table 11.1. III).

Qualitative abnormalities of protein 4.1 have been reported (see Section 11.3.3). A typical case is a shortened type of protein 4.1 (protein 4.1^{68/65}). The patients with this abnormality exhibit common hereditary elliptocytosis with moderate chronic hemolysis. The protein 4.1 content in the red cell membranes decreases to 50 % of the normal with two abnormal faint bands of 65 and 68 kDa in electrophoretic mobility. In these patients, the binding capacity of the abnormal protein 4.1 to normal spectrin and actin was reduced to approximately 40 % of normal. This shortened form of protein 4.1 is caused by a deletion of the entire 10 kDa spectrin–actin binding domain (codons 407 to 487), resulting in a deletion of 80 amino acids.

Another qualitative anomaly is a large (95 kDa) variant of protein 4.1 (protein 4.1⁹⁵). This variant is caused by the internal duplication of a 369 base pair segment consisting of three exons that encode lysine in codon 407 to glutamine in codon 529, resulting in duplication of the spectrin–actin binding domain. This additional insertion of the spectrin–actin binding domain does not produce severe hemolysis, but exhibits its mild phenotype.

Protein 4.1 Presles is a shortened protein 4.1 which demonstrates a doublet of protein 4.1 with apparent sizes of 73 and 74 kDa. This anomaly is caused by skipping one exon that encodes 34 amino acids near the beginning of the C-terminal 22/24 kDa domain, but is asymptomatic even in the homozygote.

In hereditary spherocytosis, no abnormalities of protein 4.1 and of the protein 4.1 gene have been reported.

In animal models of protein 4.1, mice lacking red cell protein 4.1 (protein 4.1R) are known [23], as well as zebrafish with a total deficiency of protein 4.1R [24]. Homozygotes by gene targeting exhibit mild to moderate hemolysis with increased fragmentation and red cell membrane instability. With a total deficiency of protein 4.1, a partial deficiency in protein p55, glycophorin C, ankyrin, and spectrin, is also observed, suggesting that loss of protein 4.1 produces an impaired membrane skeleton assembly, as observed in human protein 4.1 deficiencies. Contrary to expectations, red cell morphology demonstrates spherocytosis but not elliptocytosis, probably because spectrin deficiency present with a total deficiency of protein 4.1 appears to be critical for this event. The targeted homozygous mice show neurological abnormalities in movement, coordination, and learning, probably due to combined impairment of neuronal isoforms of erythroid protein 4.1 by the gene targeting.

14.3.3

Total Deficiency of Protein 4.1: Protein 4.1 (–) Madrid

Homozygous 4.1 (–) HE is extremely rare. The allele Madrid was identified as a point mutation of the downstream translation initiation codon (AUG→AGG) that induced the missing protein 4.1 in a homozygous state [25]. Just as in deletion experiments in nature, the homozygous 4.1 (–) Madrid case may serve as an excellent model for elucidating the crucial roles played by protein 4.1 in association with skeletal proteins in the skeletal network, in interactions with band 3, which are still controversial, and in its competition with ankyrin [26].

In this splenectomized patient, therefore, the skeletal network was examined by EM using the quick-freeze deep-etching (QFDE) method and the surface replica (SR) method with the immunogold method (see Section 3.2.2). The intramembrane particles (IMPs) were also examined by EM using the freeze fracture (FF) method (see Section 3.2.3).

Scanning electron microscopy demonstrates marked anisocytosis, poikilocytosis with elliptocytosis [26], and fragmented red cells in the 4.1 (–) Madrid (Fig. 14.9), compared with discocytes in a normal subject. Some of these changes appear to be modified by splenectomy.

Because the symptomatology was essentially restricted to the red cells, two cDNA regions were studied which appear critical to this cell type, namely motif I and the downstream initiation codon [23]. The sequence of motif I was normal. After RT-PCR amplification of 4.1 mRNA around the downstream initiation codon, a prevalent fragment of 990 bp was obtained, corresponding to the major mRNA 4.1 erythroid isoform. Sequencing, carried out after an additional set of (asymmetrical)

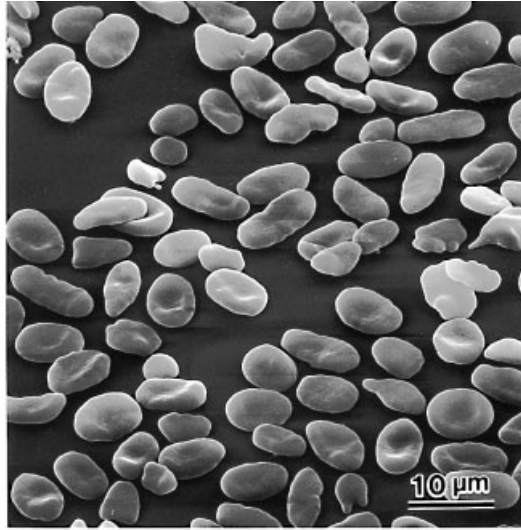


Figure 14.9 Scanning electron micrograph of peripheral red cells in the homozygous patient with total deficiency of protein 4.1 [protein 4.1 (–) Madrid].

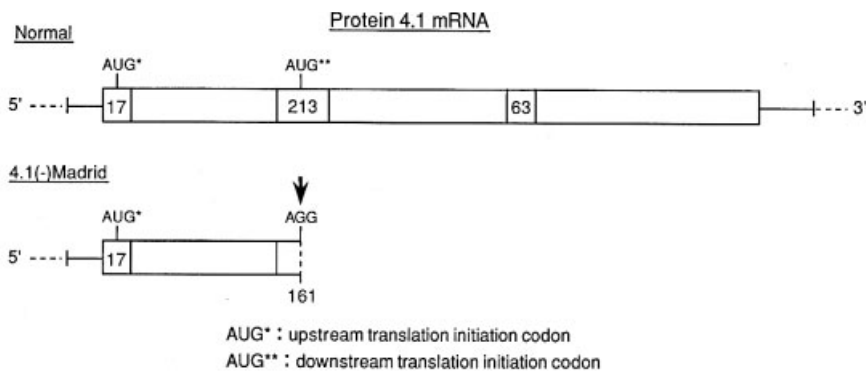


Figure 14.10 Schematic demonstration of gene mutation in protein 4.1 (–) Madrid.

PCR, showed a T to G substitution (AUG→AGG) at the position 161 of the 213nt exon. Therefore, the downstream AUG initiation codon appeared closed (Fig. 14.10).

The total absence of protein 4.1 was confirmed by SDS–PAGE and Western blotting with anti-protein 4.1 polyclonal antibodies [25]. A 55 kD protein (p55) was missing in the region of band 4.5, and glycophorins C and D were sharply diminished. The amount of spectrin (α chain and β chain) was significantly decreased by 21.4% on SDS–PAGE using Fairbanks gels. The amount of actin was also diminished by 18.4% on the Laemmli gels. The protein 4.2 content appeared to be lower than that of normal controls as judged by the Laemmli gels, but the difference was not significant. The amount of ankyrin appeared to be normal on the Fairbanks gels under the usual steady-state conditions with essentially normal reticulocyte counts ($43 \times 10^9 \text{ L}^{-1}$).

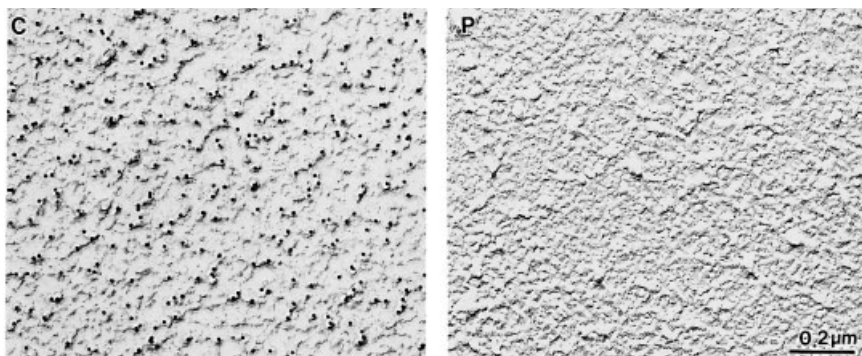


Figure 14.11. Total lack of protein 4.1 molecules in red cells of homozygous protein 4.1 (–) Madrid examined by immuno-electron microscopy with the surface replica method with anti-human protein 4.1 mouse monoclonal antibody. C: a normal subject, P: a patient.

Red cell ghosts were examined by immuno-EM using the surface replica method with anti-protein 4.1 antibody conjugated with immunogold particles. The immunogold particles (protein 4.1) were missing completely in the red cells of protein 4.1 (–) Madrid [26] (Fig. 14.11), in contrast to the normal subjects, in whom the immunogold particles with the anti-protein 4.1 antibody were found to be present normally by colocalizing at the skeletal network. In normal subjects, the number of immunogold particles with anti-protein 4.1 antibody was $186 \pm 29 \mu\text{m}^{-2}$. Therefore, the total deficiency of protein 4.1, which had been suggested by biochemical analyses on SDS–PAGE was demonstrated morphologically by the immuno-EM.

Skeletal networks of red cell membrane ghosts were examined by EM using the QFDE method [26] (Fig. 14.12). In the normal subjects, the filaments (mostly spectrin, as identified by immuno-EM) of the intact skeletal network were present in multistereotactic dimensions rather than in a single plane. The filaments in the normal subjects were $48 \pm 9 \text{ nm}$ in length and $7 \pm 1 \text{ nm}$ in diameter and appeared to be in a folded configuration. The skeletal network in normal red cells showed a fairly uniform distribution of filamentous structures and also uniformity of apparent branchpoints of the filamentous elements in an essentially orderly fashion. The skeletal network in normal subjects showed numerous basic units, resembling cages, the number of which was $548 \pm 39 \mu\text{m}^{-2}$. These cage-like structures consisted essentially of two major types of units; i. e., small (20 to 44 nm) and medium (45 to 68 nm) sized units as determined by the interdistance (or diameter) of the longer axis of each structure. In normal subjects, two-thirds of these units were of a small size ($70 \pm 10\%$), and the remaining one-third were of medium size ($25 \pm 6\%$). There were only a few large-sized units ($5 \pm 1\%$) in the normal subjects.

In contrast, with a total deficiency of protein 4.1, the uniform distribution of filamentous structures was lost, and apparent branch points of the filamentous elements were markedly disrupted or distorted. The abnormality of the skeletal network in protein 4.1 deficiency was quantitated by counting the number of apparent skeletal units still left as nearly recognizable and acceptable for these counting pro-

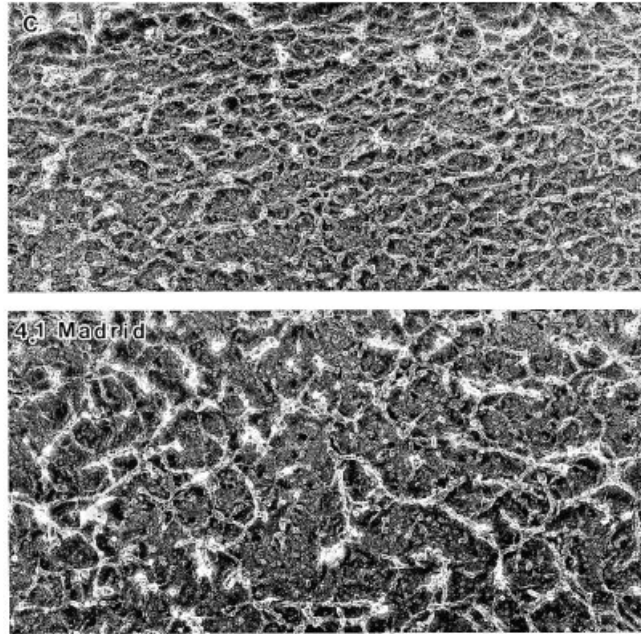


Figure 14.12 Skeletal networks examined by electron microscopy with the quick-freeze deep-etching method. Markedly disrupted skeletal networks are shown representatively in the protein 4.1 (–) Madrid (the lower panel) as compared with normal subjects (the upper panel).

cedures. The number of these skeletal units was markedly reduced in the 4.1 (–) Madrid case ($131 \pm 21 \mu\text{m}^{-2}$) [26].

The relative size distribution of these skeletal units was also quantitated by measuring the interdistance (or diameter) of the longer axis in each unit. In the 4.1 (–) Madrid case, the skeletal units of basic small size (20 to 44 nm) were markedly reduced ($17 \pm 4 \mu\text{m}^{-2}$) [26] compared with the number in the normal subjects ($384 \pm 52 \mu\text{m}^{-2}$). In their place, units of large size (69 to 92 nm) and of extra-large size (93 to 240 nm), which were rarely observed in the normal subjects ($5 \pm 1\%$), were markedly increased in the 4.1 (–) Madrid case ($64 \pm 14\%$) [26]. It should be noted that the structures being called small, large, or extra-large cages may be composed of entirely different components (and/or the same components organized differently in relation to one another) in the 4.1-deficient sample compared with the normal sample.

Intact red cells of normal subjects and of the 4.1 (–) Madrid case were examined by EM with the surface replica method [26]. The skeletal networks were further labeled by the immunogold particles conjugated with antispectrin polyclonal antibody. The filaments of the skeletal meshwork *in situ* were identified as spectrin by immunogold labeling (Fig. 14.13). The immunogold particles (spectrin) in the normal subjects were virtually evenly distributed representing a normal orderly arranged skeletal network as observed by EM with the QFDE method. The number of immunogold particles (spectrins) was $504 \pm 36 \mu\text{m}^{-2}$ in the normal subjects. In contrast, the immunogold particles for spectrin labeling in the 4.1 (–) Madrid case were definitely distributed in an uneven fashion, mainly by forming large

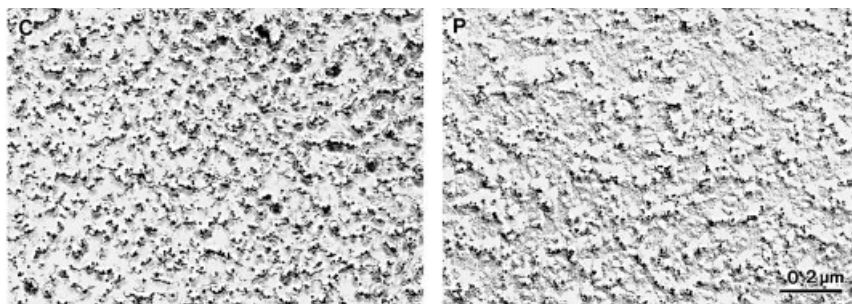


Figure 14.13 Immuno-electron microscopy of red cell membrane skeletons using the surface replica method with antihuman spectrin rabbit polyclonal IgG antibody. Aggregated spectrin was noted in the protein 4.1 (–) Madrid case (P) as compared with normal subjects (C).

clusters, or by lining up close together in a necklace fashion. It was evident that many areas of the membrane plane remained open and free from any immunogold particles, indicating that the skeletal meshwork was completely disrupted or distorted in the absence of protein 4.1. The findings were nearly identical to those by EM using the QFDE method. The number of immunogold particles was slightly diminished to $395 \pm 63 \mu\text{m}^{-2}$ (–21.6 of the mean value of normal controls) in this patient, which was compatible with the biochemical results [26].

Immuno-EM with the anti-ankyrin antibody was also applied to normal cells and the 4.1 (–) Madrid red cells. Ankyrin was almost evenly distributed in the normal subjects. The number of immunogold particles (ankyrin) was $150 \pm 34 \mu\text{m}^{-2}$ in normal subjects ($n = 20$). In contrast, large open areas without immunogold particles (ankyrin) were widely present in the 4.1 (–) Madrid case. Although the number of immunogold particles of ankyrin appeared to be slightly diminished to $123 \pm 21 \mu\text{m}^{-2}$ (–19.8 % of the normal controls), the most striking feature was the rather clustered distribution of immunogold particles [26], which were still attached to the basic units of the skeletal meshwork. There was a minor discrepancy in the ankyrin quantitation by SDS–PAGE (the same as the normal control) and by the immunogold method [slightly less in the 4.1 (–) Madrid]. This might be due to the experimental conditions, in which the ankyrin labeling *in situ* was abnormal in the 4.1 (–) Madrid probably because of the limitation of the epitopes for ankyrin molecules.

Intact red cells were subjected to EM using the FF method [26] (Fig. 14.14). In the normal subjects, the number of IMPs at the inner (so-called “P”) face was $5390 \pm 420 \mu\text{m}^{-2}$, most (71 ± 8 %) of which were basically small (4 to 8 nm) in size. In the red cells of the protein 4.1 (–) Madrid case, the number of IMPs present was normal ($5275 \pm 329 \mu\text{m}^{-2}$). The size distribution of IMPs appeared to be unaffected; i. e., 68 ± 9 % of small size (4 to 8 nm in diameter), 29 ± 5 % of medium size (9 to 20 nm), and 3 ± 2 % of large size (>21 nm). Therefore, no quantitative IMP abnormalities were observed in this patient. However, there was a striking disappearance of the regular distribution of IMPs of small size that were a major component of IMPs [26]. In the patient, the IMPs appeared to form clusters of various

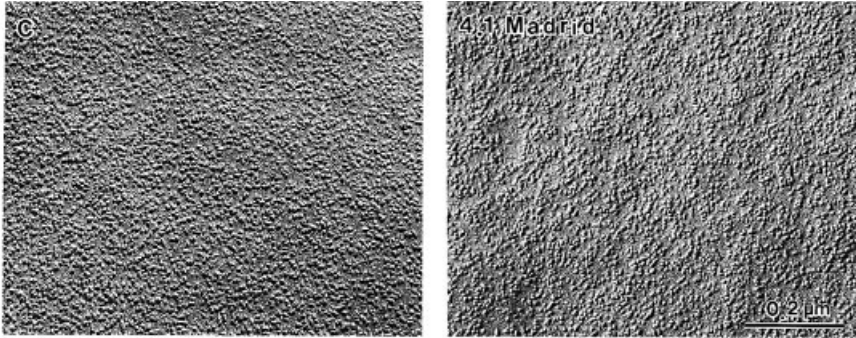


Figure 14.14 Intramembrane particles at the inner (so-called “P”) face by electron microscopy with the freeze-fracture method. No quantitative abnormalities were detected in the protein 4.1 (–) Madrid case (right), as compared with normal subjects (C: left). However,

regular distribution was lost with many clusters of various sizes in the protein 4.1 (–) Madrid case. Many irregularly and widely open membrane areas with a much smaller number of IMPs were also observed.

sizes that were composed of 3–10 IMPs. The uneven distribution of IMPs at the inner (so-called “P”) face in the patient was demonstrated by counting the numbers of IMPs in surface areas of a specific size by EM using the freeze fracture method [26]. In normal subjects, approximately 80% of each membrane face area contained 6–11 IMPs per 33 nm^2 . Only 2.4% of the membrane areas contained 0–3 IMPs per 33 nm^2 . In contrast, in the 4.1 (–) Madrid red cells, nearly 40% of the membrane face areas contained 0–5 IMPs, although the total number of IMPs was nearly identical on a larger scale (i.e., per square micrometer).

Protein 4.1 is known as one of the most important proteins because of its interactions with spectrin, actin, and integral proteins in the lipid bilayer (see Section 4.2.2). It has been shown that protein 4.1 binds tightly to β -spectrin very near to the actin binding site, probably within the N-terminal domain, and also that its 10 kDa domain strengthens the spectrin–actin binding. Using EM, it has been shown that protein 4.1 and actin bind at the end of the β -spectrin molecule. The ternary complex appears to be regulated by the degree of phosphorylation by protein kinase A, by tyrosine kinase, and by Ca^{2+} and calmodulin. Protein 4.1 also appears to have binding sites for band 3, glycophorin A (GPA), and glycophorin C (GPC), probably at its 30 kDa domain. Protein 4.1 deficient red cells have also been reported to be deficient in GPC and p55, but not in GPA or band 3. Protein 4.1 also interacts with myosin at its 10 kDa domain.

However, most of the findings described above have been obtained by *in vitro* experiments. Therefore, it should be clarified whether these interactions of protein 4.1 with other membrane proteins actually occur in the membrane structure *in situ*. If so, the primary deficiency of protein 4.1 should give rise to a tremendous disruption of the skeletal network, resulting in severe hemolysis.

A particular Spanish patient had been shown to be a homozygote of complete protein 4.1 deficiency due to a point mutation at the downstream translation initiation

codon (ATG→AGG) of the protein 4.1 gene, leading to a total lack of red cell protein 4.1. Therefore, this patient was studied by EM using the surface replica method (see Section 3.2.2.3), which was combined with the immunogold method using the anti-protein 4.1 antibody, because this procedure using the antispectrin antibody had been successfully applied to clarify the impaired skeletal network in β -spectrin Le Puy [21]. In this homozygous 4.1 (–) patient, immuno-EM proved that protein 4.1 was totally missing, as expected from the biochemical and genetic data.

The replica method with the QFDE method for EM is believed to provide the best resolution for visualization of the *in situ* condition of the skeletal network. With this method, the intact structure of the skeletal network was clearly shown in normal RBC membranes. However, in the 4.1 (–) Madrid case, the skeletal meshwork was totally disrupted or distorted, as shown by the disappearance of normal intact basic units, elongation of fibrous filaments, and disconnection of each basic unit of the skeletal network, although the amount of spectrin was only minimally decreased biochemically. In addition, no functional abnormalities of spectrin, such as the dimer–dimer association, were detected. Therefore, the marked abnormalities of the skeletal network appear to be derived from the total protein 4.1 deficiency due to the primary genetic lesion, because other proteins, especially anchoring proteins such as ankyrin and protein 4.2, appeared to be nearly normally maintained. The marked abnormalities in the 4.1 (–) Madrid case as detected by EM with the QFDE method were confirmed by immuno-EM with the surface replica method using the antispectrin antibody.

It has been proposed that the skeletal network, which is composed mostly of spectrin, is linked to the integral proteins, such as band 3, glycophorins, and others, via interaction with anchoring proteins (see Sections 4.1, 5.1 and 6.1). A critical role for protein 4.2 in connecting the skeletal network with the integral proteins (especially band 3) has recently been suggested from EM studies of red cells with a total deficiency of protein 4.2. Possible interaction of protein 4.2 with spectrin has been reported directly by a binding assay of protein 4.2 to spectrin. In this 4.1 (–) Madrid case, the amount of protein 4.2 present was nearly normal and not deficient.

Ankyrin has been reported to be one of the major anchoring proteins connecting the skeletal network (especially spectrin) to the integral proteins (especially band 3) (see Section 6.1). Therefore, it would be interesting to know whether the skeletal network could normally be supported by the presence of ankyrin even in the total absence of protein 4.1. The amount of ankyrin present in this splenectomized patient with a normal reticulocyte count was nearly normal, but the disruption of the skeletal network was extremely marked, indicating that the critical role played by protein 4.1 in construction of the skeletal network cannot be taken over by ankyrin. In addition, under conditions with a total absence of protein 4.1, ankyrin tended to cluster, as shown by the immuno-EM with the anti-ankyrin antibody. The degree of clustering of ankyrin appeared to be more marked than that of spectrin. This may imply that, in addition to the primary abnormalities of the skeletal network itself, the distribution of ankyrin was more directly affected in the absence of protein 4.1.

Finally, the interaction of the skeletal network and the integral proteins (especially band 3) under the total deficiency of protein 4.1 must be discussed. It has also been speculated that protein 4.1 may play a biological role in connecting the skeletal network to the integral proteins (especially band 3) in the lipid bilayer, although there is much controversy regarding this possibility. Surprisingly, the EM with the FF method showed no quantitative abnormalities in IMPs in the 4.1 (–) Madrid case [26], unlike the markedly decreased p55 and GPC, although protein 4.1 has been reported to be bound to band 3. The number of IMPs in this patient was normal, corresponding to the biochemical results, which showed a nearly normal band 3 content on SDS–PAGE. The sizing of the IMPs was also identical to that of normal subjects [26]. The sizing of the IMPs is considered to be one of the typical indices for determining the extent of oligomerization of band 3. Therefore, it appears evident that protein 4.1 has no substantial effect on the oligomerization of band 3, although this interpretation may be conservative, because of the fact that the number of copies of band 3 (approximately 1 000 000 per red cell) is so much greater than that of protein 4.1 (approximately 200 000 per red cell) to appreciate significant differences in the oligomeric state of band 3.

However, the most striking feature of the abnormalities in the IMPs was their uneven distribution [26]. It has been reported that band 3 consists of a mobile fraction (one-third) and an immobile fraction (two-thirds), which is fixed to the skeletal network mostly by ankyrin. Therefore, a condition involving the marked disruption of the skeletal network with clustering of spectrin and ankyrin should readily affect the state of distribution of band 3, resulting in an abnormal distribution pattern of IMPs. When examined using a smaller scale (33 nm²), some membrane areas should contain the clustered IMPs, which should be composed mostly of the immobile band 3 attached to the distorted skeletal network and/or of the mobile band 3 trapped in collapsed compartments of the skeletal proteins. However, other areas should contain a much smaller number of IMPs. The abnormal distribution pattern of the IMPs in the 4.1 (–) Madrid case, therefore, appears mainly to reflect the markedly impaired skeletal disruption.

Another consideration is a possible competitive interaction between ankyrin and protein 4.1. Ankyrin has its binding to band 3 near the N-terminus and also at a putative central hinge. Protein 4.1 also has its binding to band 3 predominately near the N-terminus and also near the junction of the cytoplasmic domain and the membrane domain of band 3. Therefore, protein 4.1 may be in competition with ankyrin with regard to its binding to band 3. In the absence of protein 4.1, ankyrin (~100 000 copies per red cell) may have more opportunities to bind to band 3 molecules, unless it has to share its binding to band 3 with protein 4.1. The increased binding of ankyrin to band 3 in the absence of protein 4.1 may enhance the clustering of band 3, which is connected to or is trapped in the collapsed compartments of skeletal proteins.

Other integral proteins, such as p55 and GPC, which were also diminished secondary to the absence of protein 4.1, might be involved in the abnormalities of the IMPs to some extent, despite the fact that there is no direct evidence.

In summary, marked disruption of the skeletal network *in situ* was shown specifically in the protein 4.1 (–) Madrid case by EM using the QFDE method and the surface replica method combined with the immunogold method using antibodies against spectrin, protein 4.1, and ankyrin. The abnormal distribution of IMPs (especially band 3) was probably mainly a reflection of the impaired skeletal network [26] (Fig. 14.15). Therefore, protein 4.1 appears to be crucial in maintaining the normal integrity of the membrane structure, both of the skeletal network and of the integral proteins.

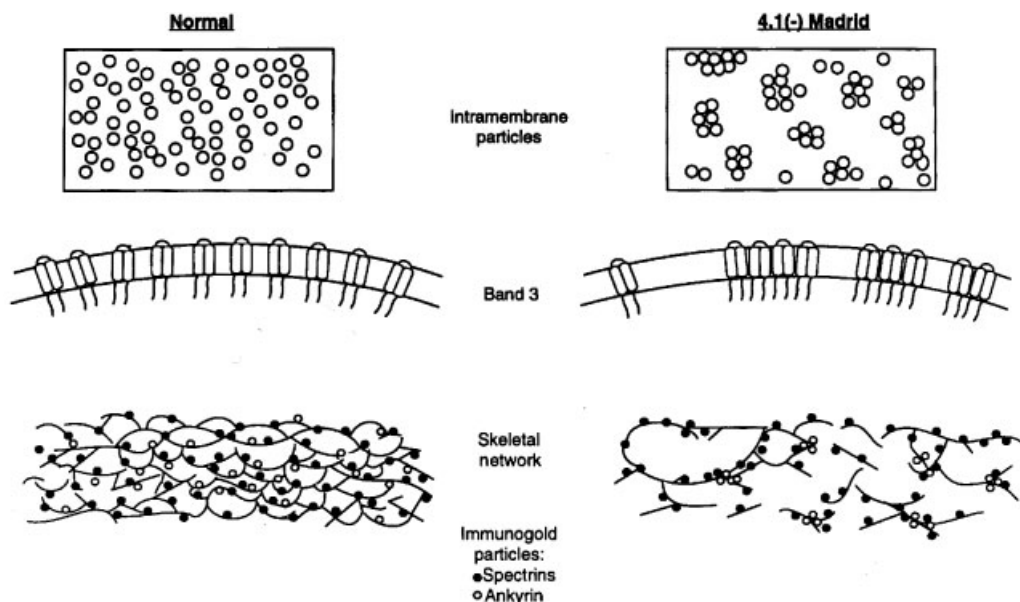


Figure 14.15 Schematic diagrams of states of IMPs and the skeletal network in the red cells of normal and the protein 4.1 (–) Madrid case. The upper and the middle portions indicate the sizes and distribution patterns in normal (left) and the protein 4.1 (–) Madrid case (right). The clustering of the normal-sized IMPs is evident in the protein 4.1 (–) Madrid case. The bottom portions represent the states of the skeletal network of normal (left) and the protein 4.1 (–) Madrid cases (right). Closed circles: anti-spectrin conjugated immunogold particles, open circles: anti-ankyrin conjugated immunogold particles. The skeletal network in the protein 4.1 (–) Madrid case is markedly disrupted, which is proven by the abnormal distribution of the immunogold particles for spectrin. Ankyrin tends to cluster more than spectrin in the protein 4.1 (–) Madrid case.

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15

Abnormalities of Integral Proteins and Blood Group Antigens

15.1

Band 3

15.1.1

Introduction

Band 3 is the anion exchanger of the red cell membrane with 1.2×10^6 molecules per cell and with 911 amino acids [1–4] (see Sections 2.3.2, 3.2.3, 5.1). This protein is a 102 kDa transmembrane glycoprotein with heterogeneous glycosylation, which demonstrates a diffuse band on SDS–polyacrylamide gels. It is divided into two structural domains; that is, the cytoplasmic domain with 403 amino acids at the N-terminal side, and the membrane domain with 508 amino acids, which form the 12 to 14 transmembrane segments. A single carbohydrate side chain is attached to the outer membrane surface at asparagine 642 carrying the I/i blood group antigens (see Section 5.3.10). The extracellular portion of band 3 also carries the antigens of the Diego blood group system (see Section 5.3.11). In addition, band 3 and glycophorin A are associated in the membrane (see Section 5.2.1.1).

The most important physiological function of the 52 kDa membrane domain is the formation of the anion exchange channel, which enables the red cells to exchange Cl^- for HCO_3^- (see Section 5.1.2.4). Much of the HCO_3^- , which is produced in red cells by carbonic anhydrase, is carried in the plasma. This channel increases CO_2 transport from the tissues to the lungs by approximately 60 %. The exchange rate for Cl^- and HCO_3^- is extremely rapid, e.g., $T_{1/2}=50$ ms. This channel is also capable of transporting larger size anions, such as sulfate, phosphate, phosphoenol pyruvate, and superoxide, although transport rates for these anions are much slower. These anions appear to be transported by a bi-directional mechanism in which an intracellular anion enters the transport channel and is translocated outwards and released, with the channel remaining in the outward conformation until an extracellular anion enters and triggers the reverse cycle. The transmembrane segments of band 3 appear to cluster together to form the transport channel. Each monomer of band 3 molecule contains a functional anion channel. CO_2 , as well as HCO_3^- , may move through this channel of band 3. Carbonic anhydrase II, which interconverts CO_2 and HCO_3^- , has its binding capacity to the C-terminus of band 3. To investigate the function of anion exchange channel of band 3, a po-

tent anion transport inhibitor (4,4'-diisothiocyanostilbene-2,2'-disulfonate: DIDS) has been widely utilized. This stilbene disulfonate binds to two lysine residues (Lys539 and Lys851) on externally exposed regions of band 3 in or near the entrance of this anion exchange channel.

The 43 kDa cytoplasmic domain at the N-terminal region of band 3 is a water soluble, 403 amino acid segment (see Section 5.1.1). Characteristic features of this domain are the presence of the hinge region, and of the binding sites for a large number of red cell cytosolic and membrane proteins (see Section 5.1.2.1). This domain stretches at higher pH and contracts at lower pH at its hinge region. The cytoplasmic domain of band 3 binds to hemoglobin, hemichromes, and glycolytic enzymes at its very acidic segment located at the extreme N-terminus. Approximately half of the band 3 molecules are bound under physiological conditions. Hemichromes, which are a partly denatured form of hemoglobin, also bind to band 3 resulting in band 3–hemichrome aggregates, which are usually known as the Heinz bodies. Regarding glycolytic enzymes, approximately 65 % of glyceraldehyde-3-phosphate dehydrogenase activity, 50 % of phosphoglycerate kinase activity, and 40 % of aldolase activity are bound in the intact red cells, probably mostly to band 3 molecules (see Section 5.1.2.2). These enzymatic activities are inhibited by this membrane attachment, which is also regulated by substrates, cofactors, inhibitors, and phosphorylation of tyrosine at codon 8 of band 3 molecule. Therefore, band 3 may be one of the important regulators of red cell glycolysis.

The cytoplasmic domain of band 3 also binds to ankyrin, protein 4.1, and protein 4.2 of red cell membranes (see Section 5.1.2.1). One ankyrin molecule binds strongly only to each band 3 tetramer. Band 3 tetramers dissociate into dimers when ankyrin is removed, and band 3 and ankyrin reassociate when ankyrin is restored. Approximately 30–40 % of the band 3 molecules are bound to ankyrin and the membrane skeleton. The binding sites of band 3 for ankyrin are the proximal, middle, and distal portions of the cytoplasmic domain. Band 3 forms stable dimers, tetramers, and higher oligomers in a solution of the nonionic detergent, octaethylene glycol n-dodecyl monoether (C12E8), which is useful for separating various oligomeric states of band 3 molecules. Normally, 70 % is in the dimeric form of band 3 and 30 % is as tetramers and oligomers. These tetramers are associated with the membrane skeleton. The band 3 molecules attached to or trapped by the skeletal network are immobile, when examined by the fluorescence recovery after the photobleaching (FRAP) method for lateral mobility. Approximately, two-thirds of band 3 molecules is mobile.

Several isoforms of band 3 are now known (see Section 5.1.3). Red cell band 3 is a member of a family of an anion exchanger (AE) gene family and is also a homologous anion transport exchanger (solute carrier family 4A: SLC4A). AE1 (or SLC4A1) is the red cell form of band 3. This AE1 is also expressed in the kidney, in which the N-terminal 66 amino acids are not included. AE2 (or SLC4A2) is the general tissue anion antiporter, and is widely distributed. AE3 (or SLC4A3) is expressed in the heart and the brain.

15.1.2

Band 3 Abnormalities

In Western countries, band 3 deficiency is thought to be one of major causes of hereditary spherocytosis [3–6] (Table 10.1 and see Section 10.4.2). According to an analysis of 166 kindred with hereditary spherocytosis by Jarolim et al. [7], deficiency of ankyrin (and its related spectrins) was found in 60 % of these kindred, band 3 in 23 %, and protein 4.2 in 2 %; 15 % were of unknown origin. Dhermy et al. [8] also reported that in 80 kindred with hereditary spherocytosis, ankyrin/spectrin deficiency was detected in 55 %, band 3 in 27 %, and protein 4.2 in 3 %; 15 % were of unknown etiology. Hassoun et al. [9] reported ten β -spectrin mutations in 40 families with hereditary spherocytosis associated with spectrin deficiency or combined spectrin and ankyrin deficiencies. These results clearly indicate that band 3 abnormalities are the second major cause pathognomonic for hereditary spherocytosis in Western countries. In contrast, abnormalities of band 3 and protein 4.2 appear to be predominant in the Japanese patients with hereditary spherocytosis [10], although ankyrin gene mutations are not particularly rare, probably accounting for one-third of all hereditary spherocytosis patients.

To date, at least 57 band 3 gene mutations have been reported in a worldwide survey (see Section 10.4.2), including 26 missense mutations, 23 frameshift and nonsense mutations, 4 splicing abnormalities, one nucleotide duplication, one in-frame deletion, and two nucleotide substitutions at the promoter region. The missense mutations are predominant, 46 % of the total band 3 mutations. The band 3 mutations in the coding regions are clustered at exons 4 and 5, 9 and 10, and 17–19. The missense mutations are also frequently observed at the cluster R region and especially at three other sites (codon 760 at exon 17, codon 808 at exon 18 and codon 837 at exon 19). The frameshift and nonsense mutations are densely localized at the region corresponding to the cytoplasmic domain of the band 3 molecule at the 5' end. The missense mutations tend to be fairly frequently localized at the region corresponding to the membrane domain at the 3' end.

The mode of inheritance of hereditary spherocytosis with band 3 mutations appears to be mostly autosomal dominantly inherited (e. g., band 3 Kagoshima [10]). The heterozygous states are usually symptomatic. In kindred with autosomal recessive inheritance, the patients are homozygotes or compound heterozygotes of missense mutations (e. g., band 3 Fukuoka [11]). Under these conditions, their parents are asymptomatic as heterozygotes.

Typical missense mutations (see Section 10.4.2) are band 3 Montefiore (E40K), band 3 Capetown (E90K), band 3 Fukuoka (G130R), band 3 Mondego (P147S), band 3 Boston (A285D), band 3 Tuscaloosa (P327R), band 3 Benesov (G455E), band 3 Yamagata (G455R), band 3 Coimbra (V488M), band 3 Bicêtre I (R490C), band 3 Dresden (R518C), band 3 Most (L707P), band 3 Okinawa (G714R), band 3 Prague II (R760Q), band 3 Kumamoto (R760Q), band 3 Hradec Kralove (R760W), band 3 Tochigi I (R760W), band 3 Chur (G771D), band 3 Napoli II (I783N), band 3 Jablonec (R808C), band 3 Nara (R808H), band 3 Birmingham (H834P), band 3 Nagoya (T837R), band 3 Philadelphia (T837M), band 3 Tokyo

(T837A), and band 3 Prague III (R870W). Frameshift and nonsense mutations are band 3 Foggia (163 del C in codon 55), band 3 Kagoshima (167 del A in codon 56), band 3 Hodouin (W81X), band 3 Bohain (241 del T in codon 81), band 3 Napoli I (298–299 ins T in codon 100), band 3 Fukuyama I (336–337 del AG or GA in codon 112–113), band 3 Osnabrück I (R150X), band 3 Lyon (R150X), band 3 Wilson (515 del G in codon 172), band 3 Worchester (515–516 ins G in codon 172), band 3 Fukuyama II (GAT→GAAT: ins A in codon 183), band 3 Princeton (823–824 ins C in codon 275), band 3 Okayama (1nt del C in codon 276), band 3 Noiterre (Q330X), band 3 Bruggen (1255 del C in codon 419), band 3 Bicêtre II (1366 del G in codon 456), band 3 Evry (1486 del T in codon 496), band 3 Milano (1498–1499 ins 69nt in codon 500), band 3 Chiba (1nt del –C in codon 526 or 527), band 3 Smichov (1848 del C in codon 616), band 3 Trutnov (Y628X), band 3 Hobart (1940 del G in codon 647), band 3 Osnabrück II (M664 del), band 3 Prague I (2464–2465 ins 10nt in codon 822), and band 3 Vesuvio (2682 del C in codon 894). Abnormal splicing mutations are band 3 Neapolis (2nt after exon 2 T→C), band 3 Nachod (3nt before exon 6 C→A in codon 117), band 3 Campinas (694 + 1 G→T in codon 204), band 3 Pribram (1431 + 1 G→A in codon 478), and band 3 Tochigi II (2058-5 del A in codon 760).

Among these mutations, conserved arginine residues in band 3 are frequent sites of mutations [12]; such as arginines in codon 150 (two mutations: band 3 Osnabrück I, and band 3 Lyon), 490 (band 3 Bicêtre I), 518 (band 3 Dresden), 760 (four mutations: band 3 Prague II, band 3 Kumamoto, band 3 Hradec Kralove and band 3 Tochigi I), 808 (two mutations: band 3 Jablonec, and band 3 Nara), and 870 (band 3 Prague III). These highly conserved residues are located mostly at the internal boundaries of transmembrane segments and substitution appears to interfere with co-translational insertion of a band 3 molecule into the membranes of the endoplasmic reticulum during synthesis of band 3. On some occasions, the mutated band 3 protein can be missing in the membrane in spite of the fact that mRNAs for both alleles are expressed, probably because the mutated protein exhibits a functional defect in its insertion into the membrane, or the mutated protein may be degraded due to its instability.

Several interesting mutations of the band 3 gene are known. Band 3 Prague I [13] (2464–2465 ins 10 in codon 822) demonstrates a ten nucleotide duplication in the reading frame and an altered C-terminus after codon 821. This mutation affects the last transmembrane helix and may eliminate the carbonic anhydrase II binding site on the band 3 molecule, resulting in an impaired insertion of band 3 into the membrane and the loss of anion transport function.

Mutations in the cytoplasmic domain of band 3, in which many binding sites are present for ankyrin, protein 4.2, hemoglobin, glyceraldehyde 3-phosphate dehydrogenase, and others, can produce a functional defect. A deletion of five amino acids from the ankyrin binding site is observed in band 3 Nachod [7] (3nt before exon 6 C→A in codon 117). A deficiency of protein 4.2 is observed in band 3 Fukuoka (G130R) [11], band 3 Montefiore [14] (E40K), and band 3 Tuscaloosa [15] (P327R), in which the cytoplasmic domains of band 3 are mutated. It is interesting to note that the proband of band 3 Fukuoka, who carry a rare homozygous missense muta-

tion of the band 3 gene, exhibit complete deficiency of protein 4.2 with a marked impairment of the binding capacity of band 3 to protein 4.2. The detailed studies are presented in the following section. The other unique case is the kindred with band 3 Okinawa [16], in whom four band 3 mutations were detected: allele Fukuoka (G130R) and allele Okinawa (K56E, P854L and G714R) *in trans*. In this proband, protein 4.2 is totally missing, disproportionately to the moderately decreased band 3 content. The mechanism of this arrangement will be discussed later in detail.

Seven types of polymorphic mutations are also detected in 42 normal individuals and in 55 Japanese hereditary spherocytosis patients [17]. In addition to previously reported polymorphic mutations, band 3 Darmstadt (D38A in exon 4), band 3 Memphis I (K56E in exon 4), a silent mutation [S438S in exon (12), and band 3 Diego (P854L in exon 19], there are three new polymorphisms which have never before been reported, i.e.: (1) band 3 Okayama (E72D in exon 5), (2) substitution A→G at the 87th nucleotide after the end of exon 7, and (3) the deletion of three nucleotides (GAG) at the 30–32 nucleotides before the start of exon 8. There are no differences in allele frequency of these polymorphic mutations of the band 3 gene between the hereditary spherocytosis patients with pathognomonic band 3 mutations (16 alleles) and those without these band 3 mutations (92 alleles).

Band 3 defects are also observed in hereditary elliptocytosis, but only in Southeast Asian ovalocytosis (SAO) [18, 19] (see Section 11.5 and Table 11.1). Two distinct mutations are detected: (1) band 3 Memphis, which is a known polymorphism, and (2) deletion of amino acid residues 400 to 408 at the junction between the cytoplasmic and membrane domains. This deletion removes part of the first transmembrane α helix, which appears to serve as an internal signalling sequence, resulting in a disruption of the structure of the membrane domain of band 3. Band 3 in the patients with Southeast Asian ovalocytosis is defective in anion transport. The affected red cells exhibit tremendously increased membrane rigidity. Band 3 in this disorder binds more ankyrin than normal, and tends to form linear aggregates in the membrane, which restrict rotational and lateral mobility of band 3 in the lipid bilayer in patient's red cells. It has also been speculated that the deletion of band 3 at residues 400 to 408 in this disease makes the flexible section of the cytoplasmic domain just proximal to the membrane more rigid, which would tend to extend the cytoplasmic domain of band 3 and cause it to become entangled in the spectrin network. This entanglement would impede the movement of spectrin chains when the membrane is stretched, leading to membrane rigidity.

Band 3 defects are associated with an incomplete distal type of renal tubular acidosis (dRTA) [20] in two patients with hereditary spherocytosis (band 3 Pribram: 1431 + 1 G→A in codon 478), although most other patients with this disorder have no evidence of metabolic acidosis. Band 3 Campinas (694 + 1 G→T in codon 204) in the patients with hereditary spherocytosis is accompanied by increased basal urinary bicarbonate excretion but efficient urinary acidification. In contrast, missense mutations at Arg589 of the band 3 gene (R589H) are observed in patients with dominant distal renal tubular acidosis but without red cell abnormality [21]. Other band 3 mutation (band 3 Coimbra: V488M) is also associated with dRTA [22]. In the other hand, band 3 missense mutations have also been found in the

patients with dRTA without red cell abnormalities [23, 24]. Thus, genotypic and phenotypic correlation on these mutations have not been elucidated precisely.

Animal models of band 3 are known in a recessive form of hereditary spherocytosis in cattle [25] with moderate hemolytic anemia and complete deficiency of band 3, due to a nonsense mutation in codon 646. In some Japanese cattle, defective anion transport, total deficiency of protein 4.2, and decreased intramembrane particles (IMPs) as detected by electron microscopy have been demonstrated. The details are given in the following section.

Mice with defects in the band 3 gene [26, 27] are also known, which are generated by targeted mutagenesis in embryonic stem cells. Mice are completely deficient in band 3 with undetectable amounts of protein 4.2 and glycophorin A, and with a severe spherocytic hemolytic anemia. In the mice, however, the contents of spectrin, actin, and protein 4.1 in their red cell membrane skeletons are essentially normal [27]. A serious vesiculation from the red cells is observed, indicating that band 3 has a crucial function in stabilizing the membrane lipid bilayer.

15.1.3

Total Deficiency of Band 3

Severe hemolytic anemia was found in nine newborn Japanese black calves [25] (P1–P9, three females and six males) in Yamagata Prefecture, Japan. The calves were weak and small (18–25 kg birth weight; normally 25–30 kg) and unable to stand or suckle by themselves at birth. They showed labored breathing, palpitations, pale and/or icteric visible mucosal membranes with increased indirect bilirubin concentrations ($2.0\text{--}6.0\text{ mg dL}^{-1}$), and splenomegaly in all cases. Hematological examination revealed severe intravascular hemolysis with significant decreases in hematocrit (Hct) values of 10–18%. All calves showed typical microspherocytosis (Fig. 15.1) with remarkably increased osmotic fragility exhibiting ~80% hemolysis even with 0.85% NaCl solution.

The proband and three affected animals survived to adulthood after 2–6 days of conservative medication, although their growth was retarded with marked jaundice and splenomegaly. The dams and siblings showed no clinical symptoms, including anemia and growth retardation. However, blood examination revealed the presence of spherocytes in peripheral blood from the dams and some siblings, although the proportions of the spheric cells were as low as 2–5%. Slightly increased osmotic fragility was observed for their red cells, particularly when examined at 24 h after blood collection. The inheritance appeared to be in an autosomal incompletely dominant mode, although the blood of sires was not available.

It should be noted that four of the nine affected animals with the total lack of band 3 survived to and thrived in adulthood (1–4 years), whereas most of the others with severe clinical symptoms died or were killed within 10 days after birth. The low viability during this period would intrinsically be due to severe hemolysis, which also occurred and subsequently disappeared in the surviving cases. Thus, some physiological factors characteristic to neonatal calves would affect the magnitude of hemolysis and survival of the animals. Factors such as genetic back-

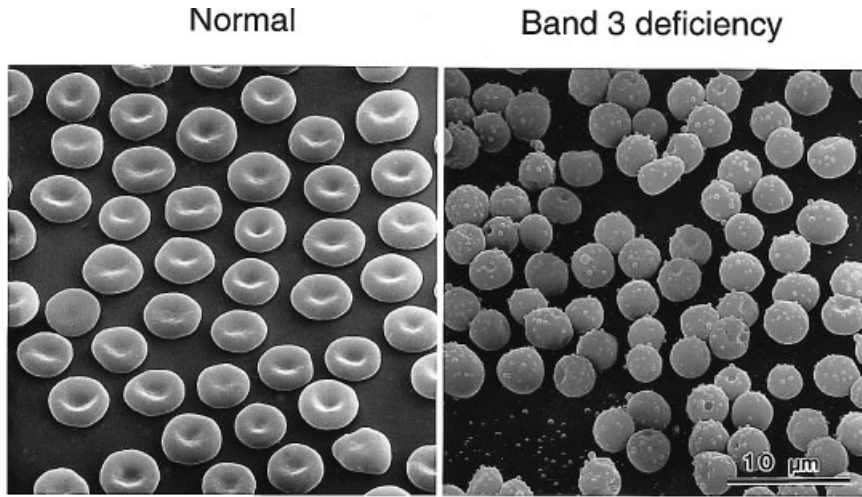


Figure 15.1 Scanning electron micrograph of bovine red cells with complete band 3 deficiency. A marked microspherocytosis with striking budding formation on the cell surface is observed in complete band 3 deficiency (right) as compared with discocytes in normal bovine red cells (left).

ground and animal husbandry, including supportive medical care at birth, also could contribute to differences in survival. It is therefore suggested that, once newborn calves survive the neonatal stage, the homozygous state of band 3 deficiency is compatible with life.

The proband showed severe Coombs negative hemolytic anemia ($Hct = 13\%$) at birth, but exhibited no clinical manifestations since one month after its birth, except for uncompensated anemia, and retarded growth. The body weight of the proband at 2 years of age was 250 kg, only $\sim 50\%$ that of normal Japanese black cattle of corresponding age. The red cell morphology of the proband resembled that of spherocytosis and anisocytosis with an elevated mean corpuscular volume and a slightly diminished mean corpuscular hemoglobin concentration. The cells constantly showed considerably increased osmotic fragility with 50% hemolysis at $\sim 0.75\%$ NaCl while the normal range was 0.48–0.55% NaCl.

Scanning electron microscopic analysis of bovine blood demonstrated that the red cells of the proband greatly varied in size, being principally spherocytic and stomatocytic with numerous small globules on their cell surface (Fig. 15.1), whereas normal cells exhibited a uniform size and biconcave disk shape. The numerous globules observed on the proband red cells were removed by washing the cells with phosphate-buffered saline at room temperature without noticeable hemolysis. The resultant stomatocytic spherocytes had irregular contours with a small number of protrusions.

The red cell membrane proteins of the proband showed a lack of the band 3 protein (Fig. 15.2). No polypeptides were immunospecifically detected in the red cell membranes from the proband. The electrophoretic profiles of the red cell membranes also revealed an apparent deficiency of protein 4.2 and considerable decreases in other major components such as spectrin, actin, and glyceraldehydes

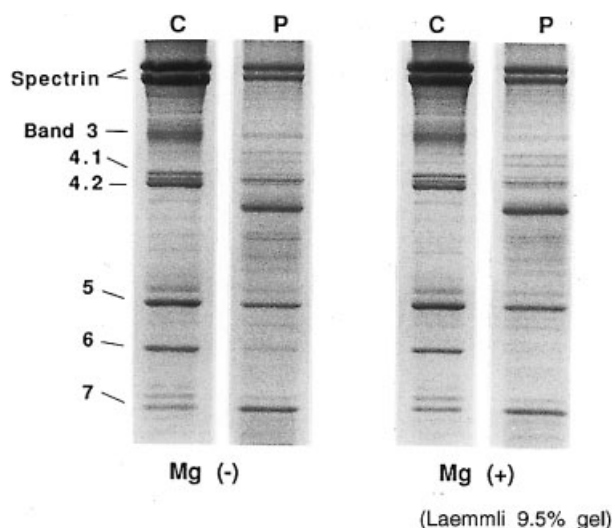


Figure 15.2 Profiles of membrane proteins on sodium dodecylsulfate polyacrylamide gel electrophoresis in bovine red cells with complete band 3 deficiency. Band 3 is totally lacking in the affected cattle (P) as compared with that in normal controls (C). It should be noted that ankyrin and protein 4.2 are also totally lacking with a significant decrease of spectrin, actin, and glyceraldehyde 3-phosphate dehydrogenase (band 6) in the proband.

3-phosphate dehydrogenase (band 6) in the proband. The content of membrane proteins in the proband red cell appeared to be as low as 60 % that of the normal cell. Immunoblotting analysis demonstrated a very low content or a nearly complete lack of protein 4.2 in red cell membranes of the proband. Moreover, there was a reduction in the ankyrin content in the proband cells by at least 50 % compared with that in normal cells. Densitometric scanning of the separated proteins showed that, in the proband red cells, spectrin (+ankyrin) and actin levels were 26 and 44 %, respectively, whereas protein 4.1 and gp155 related polypeptides were 87 and 78 %, respectively, of those in normal cells. A 66 kDa protein, comprising ~10 % of the total proteins, was found only in the sick animal and was proved by immunoblotting to be bovine albumin. The affected animals lacked the kidney protein, which is antigenically related to band 3, as in the erythroid cells from the bone marrow.

Markedly reduced numbers of intramembrane particles (IMPs) were observed on electron microscopy (EM) by the freeze fracture method (see Section 3.2.3) (Fig. 15.3). The numbers, sizes, and distribution patterns of the IMPs in normal controls appeared to be perfectly intact. In the proband, the number of IMPs was decreased by ~70 % ($1856 \pm 226 \mu\text{m}^{-2}$) compared with that of normal cattle ($5373 \pm 292 \mu\text{m}^{-2}$) in which band 3 is a major constituent of the IMPs. Therefore, the IMPs present in the protoplasmic face in the sick animal appeared to be some intramembrane components rather than band 3, i. e., various glycoproteins, such as gp155, glycophorins.

Sequencing analysis of a portion of these PCR-amplified fragments showed a C→T substitution in the proband cDNA, resulting in a nonsense mutation

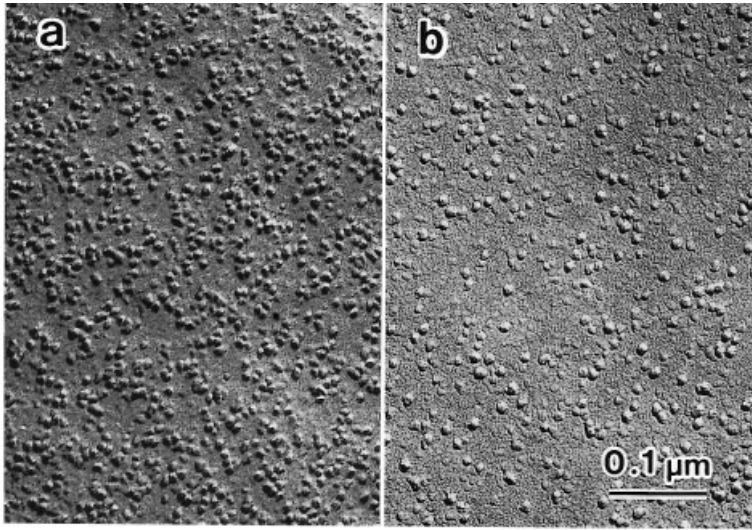


Figure 15.3 Markedly reduced number of IMPs accompanying bovine red cell band 3 deficiency by electron microscopy with the freeze fracture method. The number of IMPs was significantly reduced in the proband red cells (b) by up to 70%, as compared with normal bovine red cells (a).

(CGA→TGA; Arg→stop) at the position corresponding to amino acid 646 in human band 3 cDNA and in their genomic DNA. This mutation localizes in the extracellular face between membrane spans 7 and 8 of red cell band 3 protein according to the secondary structure prediction. However, the mutant protein, which was expected to be 70–80 kDa, was not detected by immunoblotting even in bone marrow cells rich in erythroid precursor cells. This may suggest that the total band 3 deficiency is caused by a rapid and complete loss of the mutant band 3 from the membrane after its synthesis or by a defective contranlational insertion of the mutant protein into the endoplasmic reticulum membrane.

In the proband red cells, Cl^- exchange was observed with a single rate constant of 0.0164. Whereas Cl^- exchange in normal bovine red cells was inhibited by 10 μM DIDS to a rate constant of 0.0064, no significant effect of DIDS was observed for the Cl^- exchange in the proband. Therefore, the flux rate of Cl^- in the band 3 deficient cells was very slow compared with that in the control cells, in which Cl^- transport via band 3 was inhibited by DIDS. The proband red cells completely lacked rapid anion exchange, the function of band 3 protein, and the defective $\text{Cl}^-/\text{HCO}_3^-$ exchange in these cells was uncompensated for and limited to a rather low level. These results indicated the existence of a system that could compensate for the anion exchange function of band 3 to some extent.

In the proband, both arterial and venous blood showed a decrease in the HCO_3^- concentration to ~75% of that in control cattle. Moreover, the proband blood showed a significantly decreased pH value compared with the normal blood (~0.15 pH units), demonstrating that the lowered HCO_3^- level due to the defective anion exchange in the red cells caused mild but chronic acidosis. Although the

total CO₂ content in the proband blood was rather less than that in normal blood, *p*O₂, *p*CO₂, and O₂ saturation values were normal. In the lungs of the affected animal, O₂/CO₂ exchange was achieved as effectively as in normal cattle.

The membrane skeletal network in normal red cells appeared to be arranged in a cobblestone pattern in an essentially orderly fashion and was composed of multiple smaller basic units connected to each other. Electron microscopic studies revealed that the basic membrane skeletal units were reasonably well extended with thinner, evenly stretched fiber filaments with well-organized junctional units. In normal subjects, the size of the units was 54 ± 14 nm (mean $\pm$ SD) on the longer axis and 23 ± 5 nm on the shorter axis. The membrane skeletal units were basically composed of thinner filaments in a folded conformation. These filaments were 48 ± 9 nm long and 7 ± 1 nm wide. Knob-like structures, which were attached to the longer, thinner filaments, were also observed. These filaments of the intact membrane skeleton were demonstrated in multistereotactic dimensions by EM using the QFDE method (see Section 3.2.2.2).

The basic membrane skeletal units in the proband red cells varied in size and were mostly distorted (Fig. 15.4). All of the fibrous filaments appeared to lose their interconnection with other filaments. Thus, the structure of the whole red cell membrane was clearly disorganized. The continuous three-dimensional network of fine filaments and small globules that had been observed in the normal control was totally disrupted by filaments of uneven length and width and a reduction in the number of intersections. The alignment of the network was disorderly.

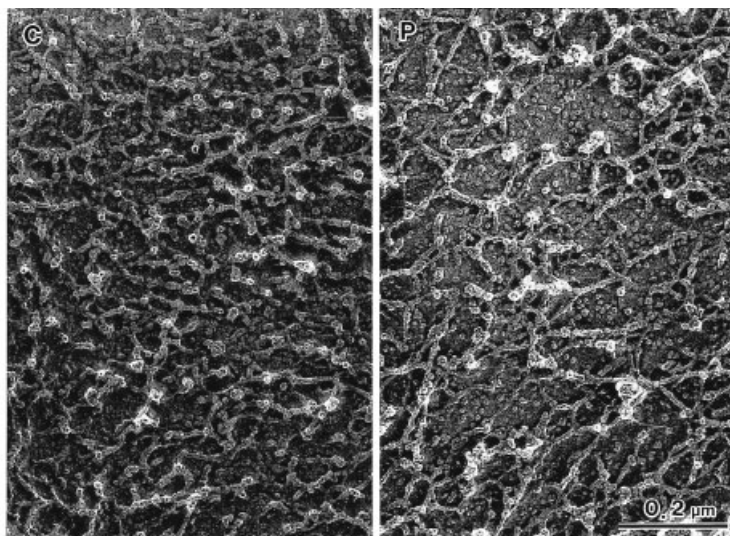


Figure 15.4 Disrupted membrane skeletal network in the proband red cells. Red cell ghosts were examined by electron microscopy with the quick-freeze deep-etching method. The membrane skeletons in the proband with band 3 deficiency were totally disrupted and distorted with filaments of uneven length and width (P) as compared with the well-organized normal red cells (C).

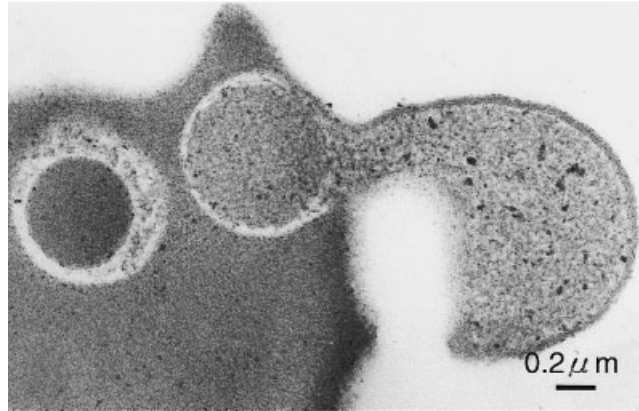


Figure 15.5 Distinct exocytosis- and endocytosis-like protrusions and projections in the proband red cells. Transmission electron micrograph indicates globule-like inclusions in the cytoplasm, in which serum albumin was detected by immunoelectron microscopy with anti-albumin antibody. A striking protrusion was detected from or along the cell surface. These observations suggest the presence of invagination or endocytosis.

EM using the surface replica method (see Section 3.2.2.3) demonstrated essentially the same results as those obtained by the QFDE method.

The proband red cells in the whole blood tended to lyse quickly if stored at 4 °C even in the presence of plasma, compared with normal cells. At 4 °C, the proband red cells demonstrated a characteristic pseudo-pod like formation as confirmed by EM with negative staining (see Section 3.2.2.1).

Transmission EM studies revealed that the proband red cells from whole blood had many small rod-like or globule-like protrusions and projections on or along the surface of these cells (Fig. 15.5), representing the vesiculation of small globules observed on these cells by scanning EM. Within these relatively larger projection (~0.5 μm in diameter), fine ultrastructures, which appeared to resemble the membrane structure, were detected. Extrusion of these microvesicles (~0.1 μm in diameter), as in exocytosis, was also observed. Vesicle-like structures were also present in the cytoplasm, which contained some amorphous or membrane-like ultrastructures, indicating that invagination or endocytosis had occurred (Fig. 15.5). These vesicles inside the cytoplasm contained bovine albumin, a plasma protein, which was occluded in them but not bound to the membrane. These observations suggested that fragmentation of the membrane occurred in the proband red cells in several distinct ways, such as invagination, vesiculation, and extrusion of microvesicles.

The fragmented vesicles were obtained as sediment after low-speed centrifugation of a hypotonic lysate of the proband red cells. The membrane protein profile in the vesicles was nearly identical to that of the original whole red cell ghosts. Therefore, α- and β-spectrins were clearly detected within the vesicle fraction on the immunoblot.

The most characteristic feature of the band 3 deficient cells was marked spherocytosis associated with fragmentation of the membrane as evidenced in a series of

electron microscopic studies. Disruption of the membrane skeletal network, directly proved by the QFDE method, corresponded well with the reduction of the membrane skeletal proteins and appeared to be the principal cause for membrane instability leading to the loss of the membrane. It is most likely that the reduction of membrane skeletal proteins was primarily due to the total lack of band 3 as their direct or indirect acceptor when these proteins were synthesized and assembled into the membrane. This is because the synthesis of band 3 only requires the membranous structure to be inserted, whereas the incorporation into the membrane of other skeletal proteins is regulated by the integral membrane proteins for their acceptors. That is, the absence of band 3 would cause a deficiency of ankyrin, which participates in the stable assembly of the spectrin heterodimer into the membrane through band 3–ankyrin–spectrin linkage in coordination with the function of glycophorin–protein 4.1–spectrin association. Hence, the spectrin–actin complex in the membrane would be decreased. Protein 4.2, which was virtually missing in the band 3-deficient cells, requires specific acceptors, such as band 3, to be assembled into the red cell membrane even if its synthesis is normal, as shown by recent studies on protein 4.2 deficiency secondary to band 3 anomalies [11, 16]. Therefore, the loss of the network organization resulting in the marked instability of the membrane was due to the total lack of the band 3 protein followed by striking reductions of spectrin, actin, ankyrin, and protein 4.2, and a consequent decrease in density of the network.

Another explanation for the network disorganization is an aberrant defect in the association of the membrane skeleton with the lipid bilayer through the band 3–ankyrin–spectrin linkage itself, which is suggested to be essential to the mechanical stability of the membrane. This is consistent with several hypothetical pathways for the surface area loss in hereditary spherocytosis proposed by Lux and Palek according to the criteria that all putative pathways share defective vertical connections between the skeleton and the lipid bilayer [28]. However, these findings showed that vesicles released or fragmented from the original red cells contained various membrane proteins, including spectrin, probably because of disorganization of the horizontal interconnection of spectrin molecules as suggested by EM with the QFDE method. This is incompatible with the observation that in spherocyte formation primarily due to partial deficiency of spectrin, ankyrin, or band 3, membrane loss occurs via a release of lipid vesicles ($\sim 0.2\text{--}0.5\text{ }\mu\text{m}$ in diameter) that contain band 3 but are devoid of spectrin. Thus, although it is still unclear whether a population of the vesicles fragmented from the proband red cells is free of spectrin, total deficiency of band 3 appears to lead to disorganization of both vertical and horizontal interconnections of membrane proteins, resulting in striking instability of the membrane. Some additional processes were also observed in the proband red cells, involving endocytic invagination and exocytic extrusion of microvesicles of $\sim 0.1\text{ }\mu\text{m}$ in diameter. These phenomena appear to be similar to the formation and the release of exosomes found in various mammalian reticulocytes. Marked instability of the membrane due to total deficiency of band 3 may lead to spherocyte formation in several distinct ways, including invagination, vesiculation, and extrusion of microvesicles.

15.1.4

Homozygous Missense Mutation: Band 3 Fukuoka

Many B3 mutations have been reported in patients with hereditary spherocytosis (HS) (Table 10.1. II).

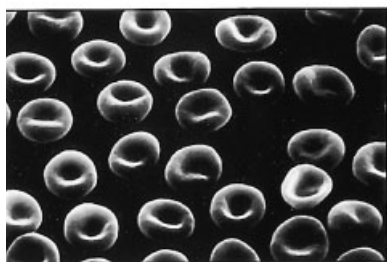
Among these mutations, frameshift mutations and nonsense mutations have been found mainly in HS patients with autosomal dominant (AD) inheritance (see Section 10.3). Missense mutations, on the other hand, have also been reported in HS patients with autosomal recessive (AR) transmission or with so-called “sporadic” occurrence, in which the mode of inheritance has not been clarified. Evaluation of such missense mutations would appear to be difficult with regard to the pathogenesis of HS itself, especially in a heterozygous state, in which an unaffected allele is clearly present in the patient in addition to a mutated allele. Therefore, the homozygous state is crucial to clarifying the significance of such missense mutations. Such a pathological condition is extremely rare, however. To date, only three definite cases have been reported: (1) band 3 Coimbra [22, 29], which has a mutation of V488M in the membrane domain of the band 3 gene, (2) the homozygous HS patient with a missense mutation in a cytoplasmic domain of the band 3 gene (codon 130 GGA→AGA: Gly→Arg in exon 6: B3 Fukuoka) [11], and (3) the homozygous HS patient with splicing mutation at position +2 in the donor splice site of intron 2 of the AE1 gene (band 3 Neapolis) [30].

Therefore, the phenotypic expressions in this homozygous proband have been studied in order to evaluate the significant contribution of this missense mutation to the pathogenesis of HS.

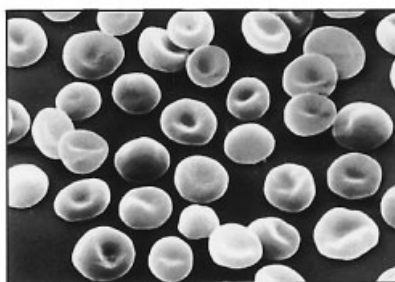
The proband (a 29 year old Japanese male) has suffered from compensated hemolysis (red cell count $4.21 \times 10^{12} \text{ L}^{-1}$, hemoglobin 13.6 g dL^{-1} , hematocrit 40.2%, MCV 96.5 fL, MCH 32.3 pg, MCHC 33.8 g dL^{-1} , increased indirect bilirubin $44 \mu\text{mol L}^{-1}$, reticulocytosis $278 \times 10^9 \text{ L}^{-1}$, and increased osmotic fragility) since birth. The red cell morphology with microspherocytosis (Fig. 15.6) was compatible with hereditary spherocytosis. This patient has not been splenectomized, and blood transfusion has not been required. His parents demonstrated normal clinical hematological findings with normal red cell morphology. The patient has no brothers or sisters.

Band 3 in the proband demonstrated a 9.3% reduction, compared with normal subjects. Quantitation of band 3 by cytofluorimetry of red cells labelled with eosin-5-maleimide confirmed this result. The ratio of band 3 tetramer to band 3 dimer was 25.6% in the proband compared with 24.4% in a normal subject, when high-performance liquid chromatography was performed. The content of protein 4.2 (P4.2) was substantially reduced in the proband (45.0% of that of in normal subjects). It is also noteworthy that, in addition to the 72 kDa peptide (a wild type of P4.2), a trace amount of the 68 kDa peptide was detected in the proband by Western blotting with anti-human P4.2 rabbit polyclonal antibody, when an excessive amount (50 μg per line) of membrane proteins was loaded. The 74 kDa peptide was not detected in the proband. In the proband, membrane, proteins other

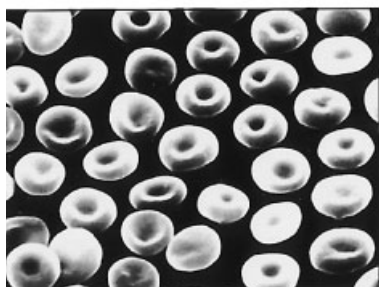
Normal



Proband (son)



Father



Mother

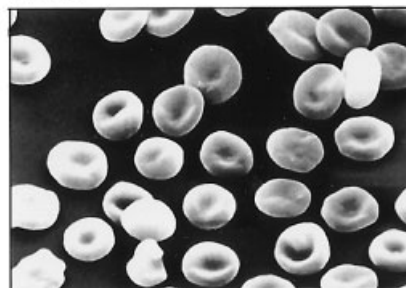


Figure 15.6 Scanning electron micrographs of peripheral red cells of a family with hereditary spherocytosis with partial deficiency of protein 4.2 (band 3 Fukuoka). A clear microspherocytosis was observed in proband (son). His parents (father and mother) demonstrated nearly normal red cell morphology, as compared with normal subjects.

than band 3 and P4.2 showed no abnormalities. No significant abnormalities were observed in his parents.

SSCP analysis of the entire coding region of band 3 cDNA [from the nucleotide (nt) -148 to nt 2815] demonstrated one point mutation with a G→A transition (GGA→AGA) at nt 388 introducing arginine (Arg) in place of glycine (Gly) at codon 130 in exon 6. This was designated as allele band 3 Fukuoka. The proband was the homozygote for this mutation, whereas his parents were heterozygotes.

For binding studies of the proband's IOVs to normal protein 4.2, pH 11 stripped IOVs were prepared from normal red cells and those of the proband. A rebinding assay of pH 11 stripped vesicles was reproducibly performed with normal protein 4.2 on three independent occasions. The extent of the rebinding of the proband's IOVs to the normal protein 4.2 was markedly reduced, compared with that of normal subjects. Scatchard plots indicated the average rebinding capacity in the proband was 207 μg of P4.2 per mg of vesicle proteins versus 295 μg in a normal subject. Therefore the rebinding capacity of the mutated band 3 Fukuoka to normal protein 4.2 appeared to be reduced to approximately 70 % of the normal band 3.

Intact red cells were subjected to electron microscopy (EM) using the freeze fracture method (Fig. 15.7). The number of IMPs was $4740 \pm 125 \mu\text{m}^{-2}$ in the proband compared with 5275 ± 329 in normal subjects. The size distribution of the IMPs in

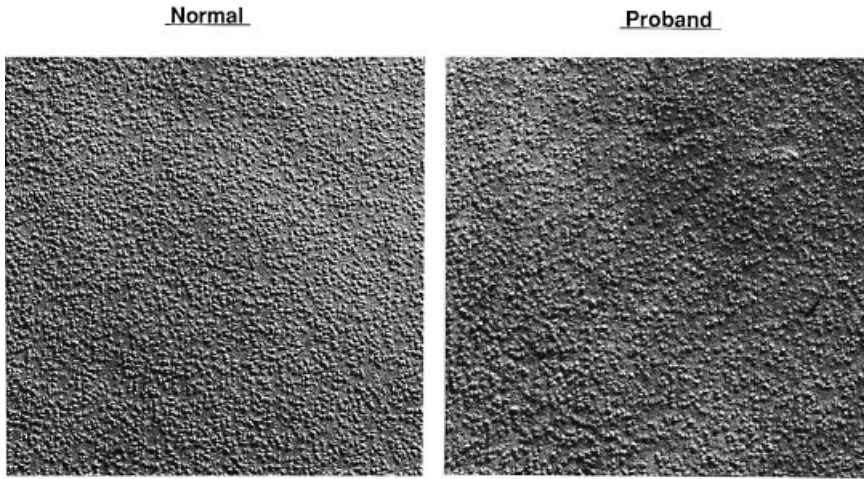


Figure 15.7 Electron micrographs of red cell membrane examined by the freeze fracture method. In the proband (right), the number of intramembrane particles was slightly diminished (-10.2%) with respect to the normal size distribution, compared with normal subjects (left).

the proband was $67 \pm 8\%$ of small size ($4-8$ nm; normal $71 \pm 8\%$), $31 \pm 4\%$ of medium size ($9-20$ nm; normal $27 \pm 3\%$) and $2 \pm 1\%$ of large size (>21 nm; normal $2 \pm 1\%$). Therefore no significant changes in IMPs were observed in the proband except for a slight reduction in number (89.8% of those in normal subjects).

Red cell membrane ghosts were subjected to EM using the QFDE method (Fig. 15.8). In normal subjects, a fairly uniform distribution of filamentous structures and also uniformity of apparent branchpoints of the filamentous elements in an essentially orderly fashion were observed. The skeletal network in the normal subjects showed numerous basic units, resembling “cages”, the number of which was $548 \pm 39 \mu\text{m}^{-2}$. In the proband, the number of basic skeletal units was $484 \pm 10 \mu\text{m}^{-2}$ ($88.3 \pm 1.8\%$ of normal) with $68 \pm 12\%$ of those of basic small size ($20-44$ nm as determined by the interdistance of the longer axis of each structure; normal $70 \pm 10\%$), $27 \pm 6\%$ of medium size ($45-68$ nm; normal $25 \pm 6\%$), and $5 \pm 3\%$ of large size ($69-92$ nm; normal $5 \pm 1\%$). Therefore the skeletal network *in situ* was almost normally maintained in the proband.

This homozygous patient with HS associated with a missense mutation (codon 130 GGA→AGA: Gly→Arg in exon 6; band 3 Fukuoka) demonstrated compensated hemolysis with disproportionately decreased (-55%) protein 4.2, compared with a mild reduction (-9.3%) of band 3. The pathogenesis is most probably due to an impaired binding capacity of the mutated band 3 protein to normal protein 4.2 as shown in band 3 Tuscaloosa and band 3 Montefiore, in which the inheritance pattern appears to be autosomal recessive. Patients are expected to demonstrate clinical hazards only in the homozygous state, which has not been well documented. In this proband, codon 130 appears to be one of the hot spots as a binding

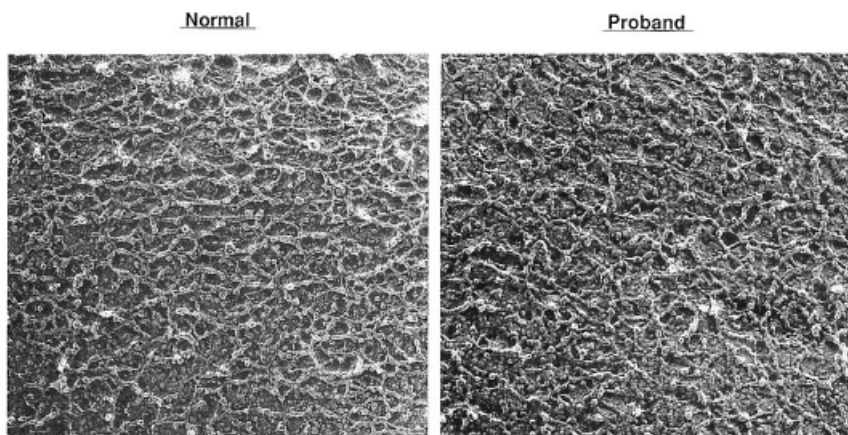


Figure 15.8 Electron micrographs of red cell membrane examined by the quick-freeze deep-etching method. The number of basic units of the skeletal network was only minimally reduced (-11.7%) with respect to the normal size distribution in the proband.

site to protein 4.2. Regarding the mutation (G130R) found in this proband, highly conserved glycine at segment 7 was replaced by arginine, which is positively charged. Therefore this mutation is truly pathognomonic in a homozygous state, but probably not in a heterozygous state, because normal hematology and protein chemistry were demonstrated in his heterozygous parents.

Although the exact role of P4.2 in the red cells has not been elucidated, many reports have recently been published regarding complete P4.2 deficiencies. The most striking phenotypic feature of the complete P4.2 deficiencies is the tremendous abnormalities in IMPs [31], that is, the decreased number, but markedly increased size, of IMPs. In addition, a marked disruption of the skeletal network *in situ* has been observed in complete P4.2 deficiencies. These findings appear to indicate that P4.2 controls the biophysical state of both band 3 and the skeletal network, which should be tightly linked to IMPs. Therefore, in the total absence of P4.2, serious derangements of red cell membranes *in situ* have been observed, not only in the integral proteins vertically, but also in the skeletal proteins horizontally.

The question then arises as to what extent protein 4.2 deficiency may cause serious damage to the red cell membrane structure. To try to answer this question, the homozygous proband with a substantial (-55%) deficiency of P4.2, which resulted from a band 3 mutation (G130R: GGA $\rightarrow$ AGA) was investigated electron microscopically.

First of all, the number of IMPs was only minimally diminished (roughly -10%), and the size distribution was almost identical to that of normal subjects. The distribution of IMPs on the membrane plane was also nearly normally maintained. On SDS-PAGE gels, the amount of band 3, which is a major component (approximately 80%) of IMPs, was only decreased by approximately 10%, even

though the patient was homozygous for the band 3 mutation (G130R). On EM, the mutated band 3 protein itself appeared to behave almost normally on the P face of the red cell membrane.

The skeletal network was also examined by EM to determine whether the network might be deranged by the decreased protein 4.2 content. The number of basic skeletal units, however, was near-normal ($484 \pm 10 \mu\text{m}^{-2}$; normal 548 ± 39). The size distribution of these skeletal units was also unaffected, and consisted mostly of units of small size ($68 \pm 12\%$, normal 70 ± 10). Therefore even decreased (to approximately 45 % of normal subjects) protein 4.2 appeared to be sufficient to maintain the normal structure of the skeletal network.

Although this patient was a homozygote of the G130R mutation of the band 3 gene, increased hemolysis was reasonably compensated for with normal red cell counts and minimal changes in red cell morphology. Neither blood transfusion nor splenectomy was required, contrary to the serious clinical pictures and striking abnormalities in red cell membrane structure in the total absence of P4.2.

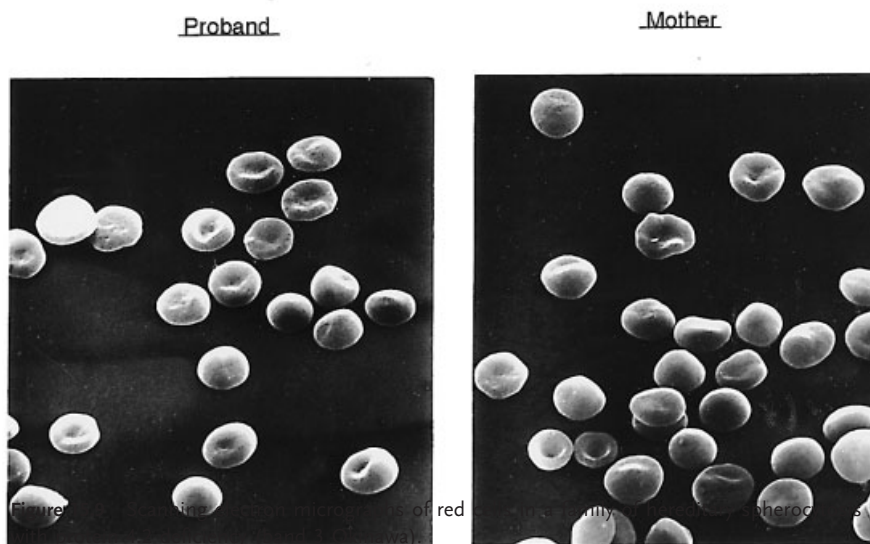
In summary, a partial deficiency of P4.2, if the level of deficiency was within approximately 45 % of normal, does not appear to be critical for maintaining the normal integrity of red cell membrane structure *in situ*, both in the integral membrane components and also in the skeletal network. Therefore the state of P4.2 appears to play a critical role in determining the whole picture of red cell membrane abnormalities.

15.1.5

Total Deficiency of Protein 4.2 Due to the Band 3 Gene Mutations: Band 3 Okinawa

These interesting band 3 mutations were observed in two related HS patients (Fig. 15.9) [16]. The mother displayed mild HS associated with band 3 deficiency. She carried a novel band 3 allele, allele Okinawa, characterized by the substitution of a single amino acid (G714R) in a conserved position of TM9 and occurring on a Memphis II allele. This mutation specifically caused HS. The daughter had a more severe HS associated with a greater reduction of band 3 and a total absence of protein 4.2. She was a compound heterozygote for allele Okinawa and allele Fukuoka (G130R), which is known to impede the binding of protein 4.2 to band 3. In the heterozygous state, allele Fukuoka shows no symptoms and presents with a normal content in band 3.

The two related family members, a mother (unsplenectomized) and her daughter (splenectomized), originated from the Island of Okinawa, Japan. The unsplenectomized mother (born in 1939) had the following red cell indices: red cell $3.16 \times 10^{12} \text{ L}^{-1}$, Hb 11.0 g dL^{-1} , Hct 30.5 %, MCV 96.3 fL , MCHC 36.1 g dL^{-1} , reticulocytes $220 \times 10^9 \text{ L}^{-1}$, indirect bilirubin $16 \mu\text{mol L}^{-1}$. Prior to splenectomy, the daughter (born in 1967) was transfusion-dependent (average 400–600 mL per year). After splenectomy with cholecystectomy in 1980, her clinical condition improved, and blood transfusions were no longer required, but her red cell indices did not normalize, e. g., RBC $3.43 \times 10^{12} \text{ L}^{-1}$, Hb 11.4 g dL^{-1} , Hct 30.8 %, MCV 89.8 fL , MCHC 37.0 g dL^{-1} , reticulocytes $65 \times 10^9 \text{ L}^{-1}$, indirect bilirubin



$4 \mu\text{mol L}^{-1}$. Scanning electron microscopy showed fewer abnormal cells in the splenectomized daughter than in the unsplenectomized mother with a marked reticulocytosis, as is often observed in HS patients after splenectomy. Based on Blood Bank standard serological procedures, the Diego blood type was identified as Di ($a^{-}b^{+}$) in the mother and the daughter.

The father was reported as clinically normal in an earlier study with the following red cell indices: red cell $4.50 \times 10^{12} \text{ L}^{-1}$, Hb 14.3 g dL^{-1} , Hct 43.6 %, MCV 96.9 fL , MCHC 32.8 g dL^{-1} , reticulocytes $95 \times 10^9 \text{ L}^{-1}$, indirect bilirubin $4 \mu\text{mol L}^{-1}$. This was in good agreement with our recent observation of another heterozygote for allele band 3 Fukuoka. The father is no longer available for further study.

Band 3 disclosed a reduction which was less pronounced in the mother than in the daughter (Fig. 15.10). In the mother, protein 4.2 was decreased roughly in the same proportion as band 3. Protein 4.2 was almost completely lacking in the daughter in whom only traces of 72, 68 and 66 kDa fragments could be seen by Western blot. The migration and levels of spectrin, ankyrin, protein 4.1, and actin were normal. Analyses of band 3 copies by cytofluorimetry with eosine-5-malimide in the daughter showed $49.8 \pm 0.3 \%$ compared with 100 % in a normal control. The protein phenotype of the father was normal.

The red cell membranes of the daughter and the mother, following proteolytic treatment with trypsin (200:1, w/w), exhibited only the 22 kDa fragment of band 3. This observation clearly indicated that the Memphis I polymorphism was not expressed at the protein level. These findings were confirmed with anti-band 3 antibody.

SSCP analysis of the coding region of band 3 cDNA was performed in the daughter. Two mutations were detected, that is, the Memphis I polymorphism (K56E) and mutation Fukuoka (G130R), *in trans* to one another. A third mutation was detected as the Memphis II polymorphism (P854L).

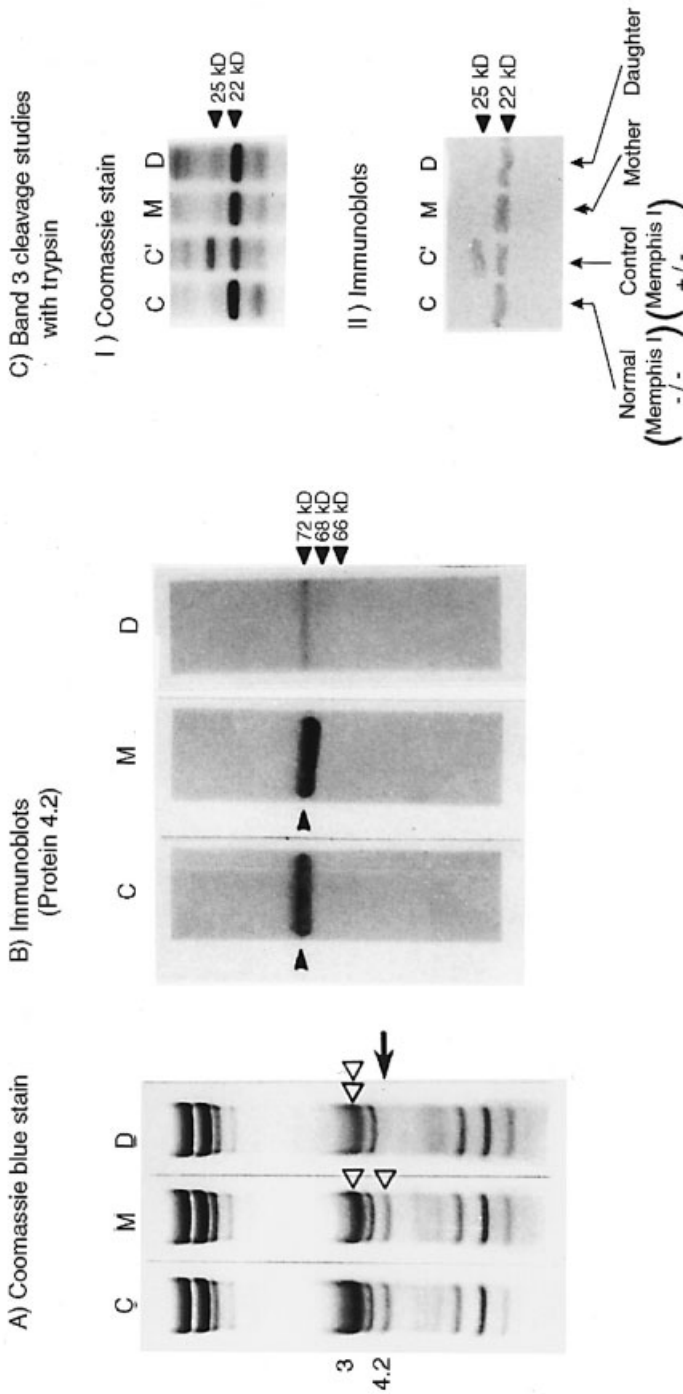


Figure 15.10 Analysis of membrane proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis and immunoblots in red cells of the patients of the Okinawa family with band 3 mutations. A, Procedure of Fairbanks et al. with Coomassie blue staining. Band 3 content is significantly reduced with nearly total deficiency of protein 4.2. B, Immunoblots with polyclonal anti-protein 4.2 antibodies. Protein 4.2 is virtually missing with the presence of protein 4.2 antibodies. C: control with a band 3 Memphis I polymorphism in the heterozygous state. M: mother, and D: daughter (proband).

Unlike previously reported cases, however, band 3 Memphis II was not incorporated into the membrane as proved by the absence of the Di^a blood group antigen and the normal digestion pattern of band 3. In the daughter, the fourth mutation G714R, GGG→AGG was detected in Memphis II cDNA. This variant of band 3 Memphis II is referred to as band 3 Okinawa.

At the gene level, the various mutations carried by allele Okinawa were confirmed, based on (i) Memphis I polymorphism (exon 4), and (ii) the presence of mutations G714R (exon 17) and P854L (exon 19) by SSCP and nucleotide sequencing. Likewise, SSCP and nucleotide sequencing confirmed the presence of mutation band 3 Fukuoka, G130R (exon 6) on the other allele. Mother and daughter shared the same three mutations on allele Okinawa. In addition, the daughter also carried allele Fukuoka.

Finally, the coding region of protein 4.2 cDNA and the 180 nt sequence upstream from the ATG initiation codon were normal.

The mother presented with mild HS associated with band 3 deficiency and a secondary decrease in protein 4.2. The band 3 HS mutation (G714R) appeared on a Memphis II polymorphic allele. Band 3 Okinawa was absent in the red cell membranes of both daughter and mother as ascertained by tryptic digestion and Diego blood group typing. The G714R mutation alters a highly conserved position in TM9. The arginine residue introduced a positive charge in a neutral amino acid stretch, which probably prevented band 3 Okinawa from being incorporated into the membranes. Other mutations of conserved amino acids pertaining to the membrane domain have been reported in association with HS and a partial deficiency of band 3.

Paradoxically, homozygosity for allele Fukuoka allowed the presence of approximately 55 % of protein 4.2 in the membrane. This suggested an important residual binding capacity of band 3 Fukuoka for protein 4.2. As a consequence, it was not expected that the Fukuoka/Okinawa compound heterozygosity would virtually prevent any binding of protein 4.2. In order to reconcile these contradictions, we put forward a hypothesis (Fig. 15.11). It takes into account the facts: (i) that band 3 is synthesized prior to protein 4.2 in erythroblasts, (ii) that an overwhelming excess of band 3 (approximately 1 200 000 copies per red cell) exists with respect to protein 4.2 (about 200 000 copies per red cell), and (iii) that there is a large cytoplasmic domain to be synthesized before band 3 initiates its incorporation into the membrane. The cytoplasmic domain of band 3 Okinawa would completely override the cytoplasmic domain of band 3 Fukuoka, binding virtually all the protein 4.2. Subsequently, the band 3 Okinawa–protein 4.2 complex would not be admitted into the membrane (G714R mutation) and would be degraded. Band 3 Fukuoka would become incorporated only with traces of protein 4.2 and some degradation products.

This series of events is reminiscent of that elicited by allele α^{LELY} , a low expression variant of the spectrin α -gene (see Section 11.3). The α^{LELY} chains have a normal self-association site, but hardly bind their β -chain partners due to a lack of six amino acids in their nucleation site at the C-terminal region of the α -chain. As a result, the unbound α^{LELY} chains are thus mostly degraded, and the α^{LELY} β dimers,

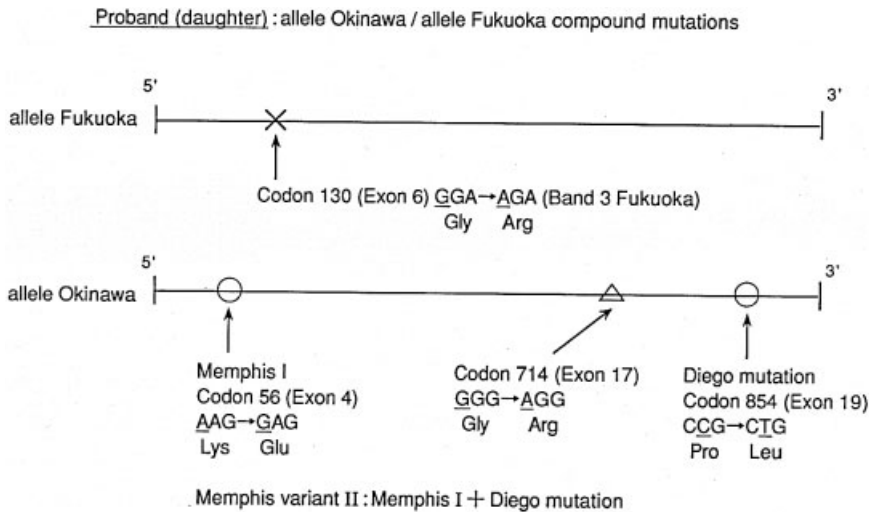


Figure 15.11 Schematic diagram of DNA analysis of the erythroid band 3 gene in hereditary spherocytosis (band 3 Okinawa) with protein 4.2 deficiency.

if any, will subsequently self-associate poorly and will be degraded. The behaviour of the band 3 Fukuoka molecules echo that of the α^{LELY} chains.

It is known that band 3 Genas (see Section 11.3) appears to cause no symptoms or biological abnormalities except for a slight reduction of band 3 content. Allele Genas produced an additive effect to those of allele Lyon, which was responsible for common HS associated with band 3 deficiency. In particular, there was a more pronounced reduction of band 3 and protein 4.2, and the reductions were roughly proportional. It can be stated, therefore, that the protein phenotype in the compound heterozygous state arose from the sum of the phenotypes in each simple heterozygous state. Likewise, the Fukuoka/Okinawa combination led to an aggravated clinical presentation and an enhanced reduction of band 3. Quite dramatically, however, almost no protein 4.2 was detected in the daughter. Instead of a parallel reduction of band 3 and protein 4.2, band 3 was decreased and protein 4.2 was unexpectedly absent.

In conclusion, the compound heterozygosity for *EPB3* alleles Okinawa and Fukuoka provide a striking example in which the protein changes are much more than the sum of the changes observed in the heterozygous state for each allele taken separately.

Based on genetic and biochemical backgrounds as mentioned above, phenotypic expressions on the red cell membranes were examined by electron microscopy. A marked clustering of intramembrane particles was detected by electron microscopy with the freeze fracture method (Fig. 15.12), indicating the increased oligomerization of band 3 molecules in this proband. Concomitantly, the cytoskeletal network was significantly disrupted, as evidenced by electron microscopy with the quick-freeze deep-etching method (Fig. 15.13), implying the instability of the cytoskeletal

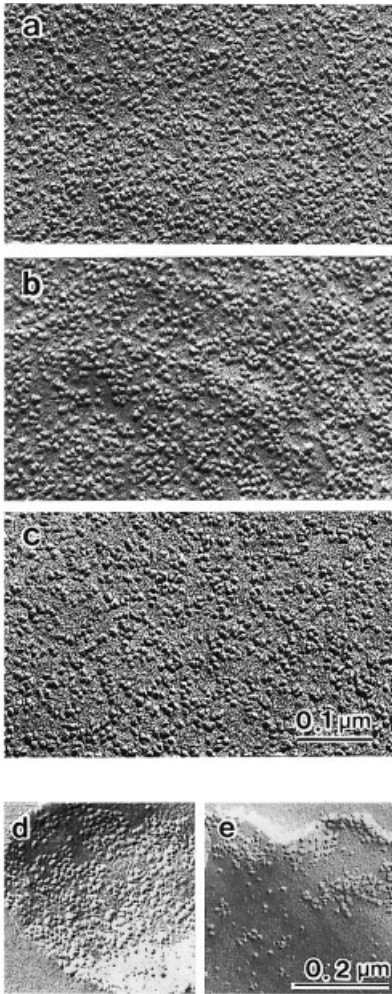


Figure 15.12 Electron micrographs with the freeze fracture method in the red cell membranes of band 3 Okinawa. a: Normal subjects, b: proband (daughter), and c: her mother. IMPs in the inside-out vesicles (IOVs) in normal subjects (d) and in band 3 Okinawa (e).

network. These observations on the ultrastructure of red cell membranes *in situ* in the proband appear to be basically due to a total deficiency of protein 4.2 in addition to the band 3 abnormality which is the principle pathogenesis in this patient. The biophysical examination of the red cell membranes in the proband by the fluorescence recovery after the photobleaching method (Fig. 15.14) demonstrated exactly the same observations as seen in the total deficiency of protein 4.2, which will be discussed in Section 16.2 (Fig. 16.10).

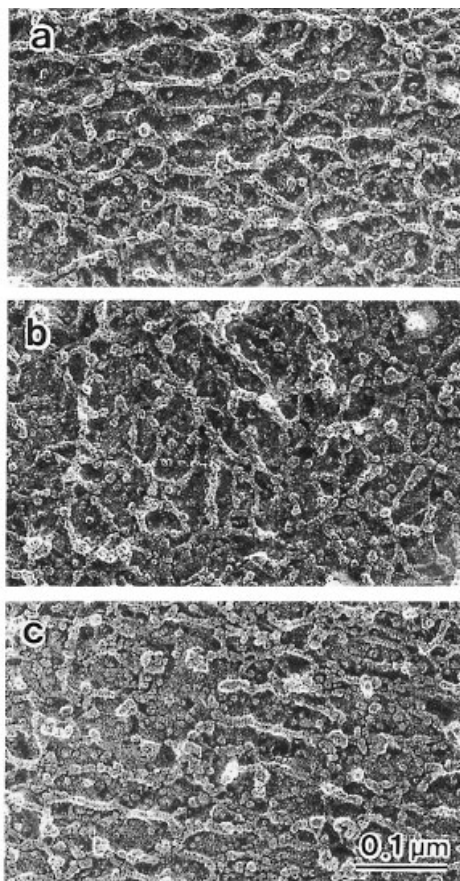


Figure 15.13 Deranged cytoskeletal networks in the patients with band 3 Okinawa examined by electron microscopy with the quick-freeze deep-etching method. a: Normal subjects, b: proband (daughter), and c: her mother.

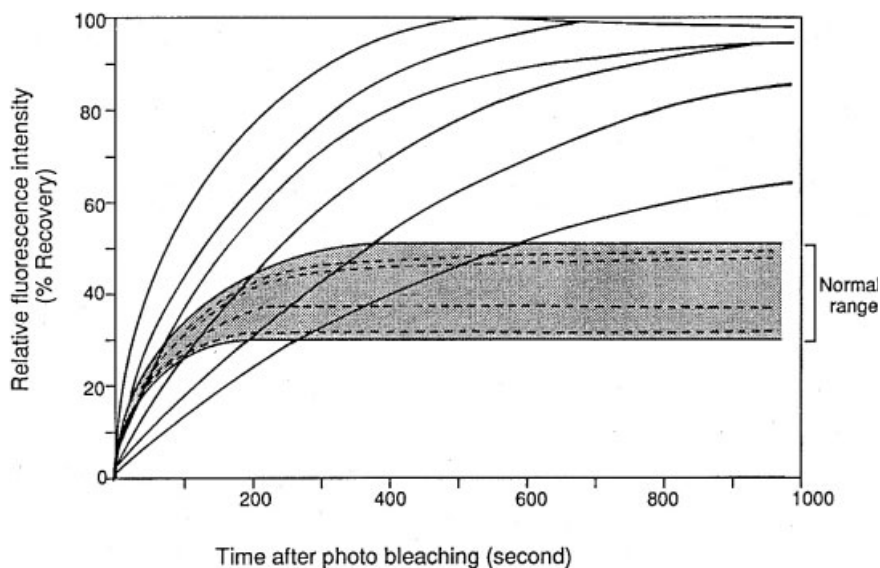


Figure 15.14 Marked increase of a mobile fraction of band 3 molecules with much slower recovery in band 3 Okinawa with the protein 4.2 deficiency studied by fluorescence recovery after the photobleaching method. A shaded area denotes a normal range.

15.1.6

Partial Deficiency of Band 3 in Hereditary Spherocytosis

Band 3, or the anion exchanger 1 (AE 1), is the most abundant protein in the red cell membrane, and is composed of 911 amino acids. The first 403 residues constitute the cytoplasmic domain to which a number of proteins bind, including ankyrin and protein 4.2. Residues 404–911 account for the membrane domain which harbors 14 membrane-spanning segments (TM). The organization of the human red cell anion exchanger gene (*EPB3* gene) has been established.

Hereditary spherocytosis (HS) is a common hereditary hemolytic anemia. A number of HS cases are due to band 3 mutations (see Section 10.4).

Two situations may be distinguished. (i) The red cells partially or completely lack one haploid set (heterozygous state) of mutant band 3 (20–40% reduction of overall band 3), yielding mild to moderate HS with a dominant inheritance pattern. Heterogeneous mutations have been elucidated. As a consequence, protein 4.2 is diminished at roughly the same proportions as band 3. Homozygous band 3 deficiencies have been described in three animal models in which protein 4.2 is absent. (ii) Protein 4.2 is sharply decreased due to mutations in the cytoplasmic domain of band 3 which presumably affects the binding site for protein 4.2, e.g.: band 3 Tuscaloosa [15], P327R, CCC→CGC; band 3 Montefiore [14], E40K, GAG→AAG; and band 3 Fukuoka [11], G130R, GGA→AGA. The inheritance pattern is recessive for

band 3 Montefiore and band 3 Fukuoka, but has not been established with certainty for the others.

In addition, the primary and almost complete absence of protein 4.2 is observed due to the occurrence in the homozygous (or the compound heterozygous) state of mutations in the *EPB42* gene encoding protein 4.2. The inheritance pattern is also recessive.

The band 3 Memphis variant II is associated with an altered binding of 4,4'-diisothiocyano-dehydrostilbene-1,2-disulfonate (H_2DIDS) due to the P854L, CCG→CTG mutation. It carries *in cis* the Memphis I and II polymorphisms, K56E, AAG→GAG. Memphis I and II polymorphisms are relatively common among Orientals. The P854L polymorphism is the structural basis of the Diego (Di^a) blood group antigen which is known to be responsible for hemolytic disease of the newborn, but not for HS.

15.2

Glycophorins

15.2.1

Glycophorin A and B Variants

Complete deficiency of glycophorin A (GPA) [32] (see Sections 5.2.1 and 5.2.2) is known as the En (a-) phenotype with no detectable MN antigens (Figs. 15.15–15.17). Two types of En (a-) variant are present. In a Finnish type [33], substantial portions of *GYP A* are absent, whereas *GYP B* gene is basically intact. Thus, the individuals of this type are due to a homozygous deletion of the *GYP A* locus. In contrast, the En (a-) phenotype in England [En (UK)] [34] demonstrates a hybrid consisting of the 5' end of *GYP A* linked to the 3' portion of the *GYP B* locus. This chimeric gene generates a hybrid glycophorin molecule composed of the NH_2 -terminal portion of an M-specific GPA linked to the $COOH$ -terminal portion of a glycophorin B (GPB) molecule with S specificity.

The En (a-) red cells compensate for the loss of surface charge by increasing the glycosylation of band 3. As a result, the surface charge is only about 20 % less than expected.

The total deficiency of GPB demonstrates red cells with the S-s-U-phenotype [35, 36]. In these individuals, red cells are deficient in GPB or express a non-glycosylated, defective form of this peptide due to large deletions of the *GYP B* locus [37, 38]. On rare occasions, the S-s-U-phenotype is observed with partial deletions of *GYP B* or an apparently normal *GYP B* structure.

Both genes of *GYP A* and *GYP B* can be deleted, known as the M^k variant [39] (Fig. 15.15). In heterozygotes of the M^k variant, red cell MN and Ss antigens are present at 50 % of the levels of the wild type. In homozygotes ($M^k M^k$), red cells totally lack detectable GPA and GPB as well as MN and Ss antigens, Wr^b determinants, and the En^a antigen [40].

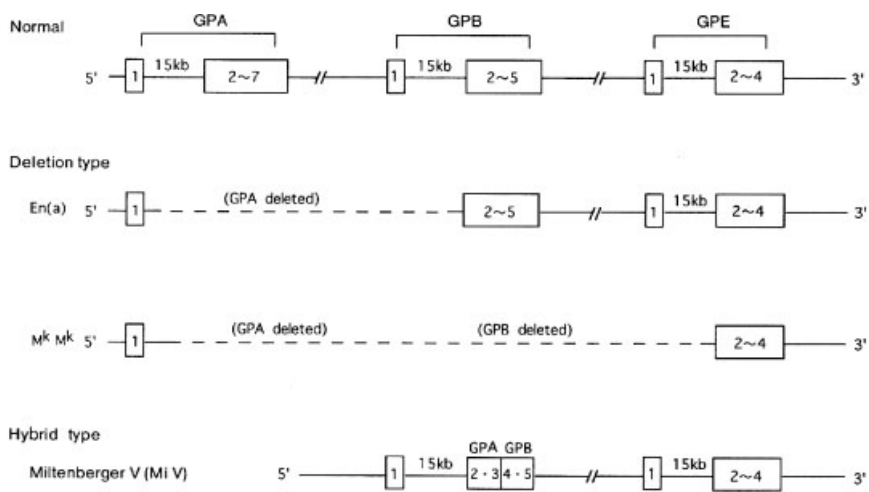
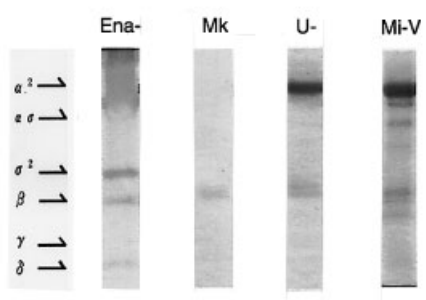


Figure 15.15 Molecular mechanism of glycophorin anomalies. GPA: glycophorin A, GPB: glycophorin B, and GPE: glycophorin E.

(A) PAS Staining



(B) Western Blotting With Anti-Glycophorin A Antibody (EnaFS:OSK4-1)

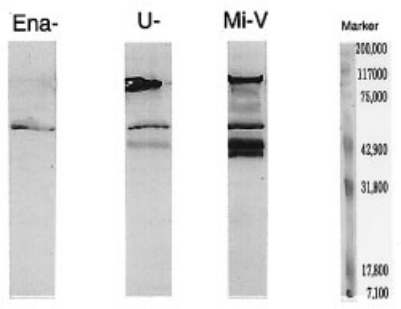


Figure 15.16 Profiles of glycophorin abnormalities in homozygotes studied by sodium dodecylsulfate polyacrylamide gel electrophoresis. (A) Periodic acid Schiff (PAS) staining, (B) Western blotting with anti-glycophorin A antibody (EnaFS: OSK-4-1). Ena⁻: total deficiency of glycophorin A, M^k: combined deficiency of glycophorin A and B, U⁻: deficiency of glycophorin B, and Mi-V: Miltenberger-V anomaly.

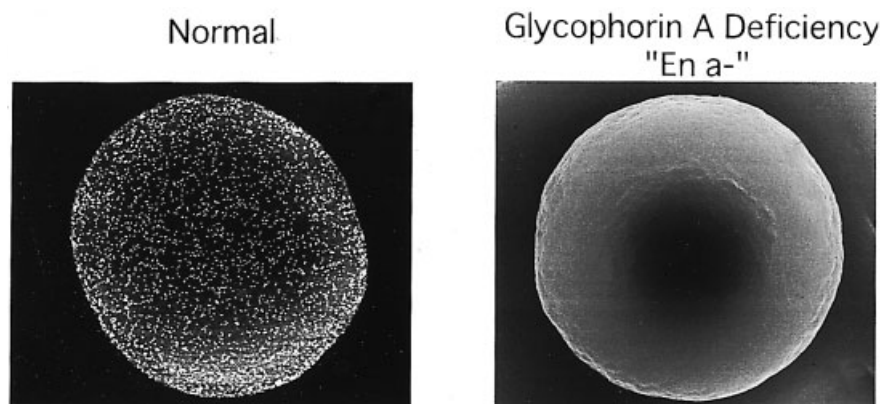


Figure 15.17 Complete lack of glycophorin A molecules in red cells of glycophorin A deficiency (En [a-]) detected by field emission scanning electron microscopy with anti-human glycophorin A monoclonal antibody (Ena FS: OSK-4-1).

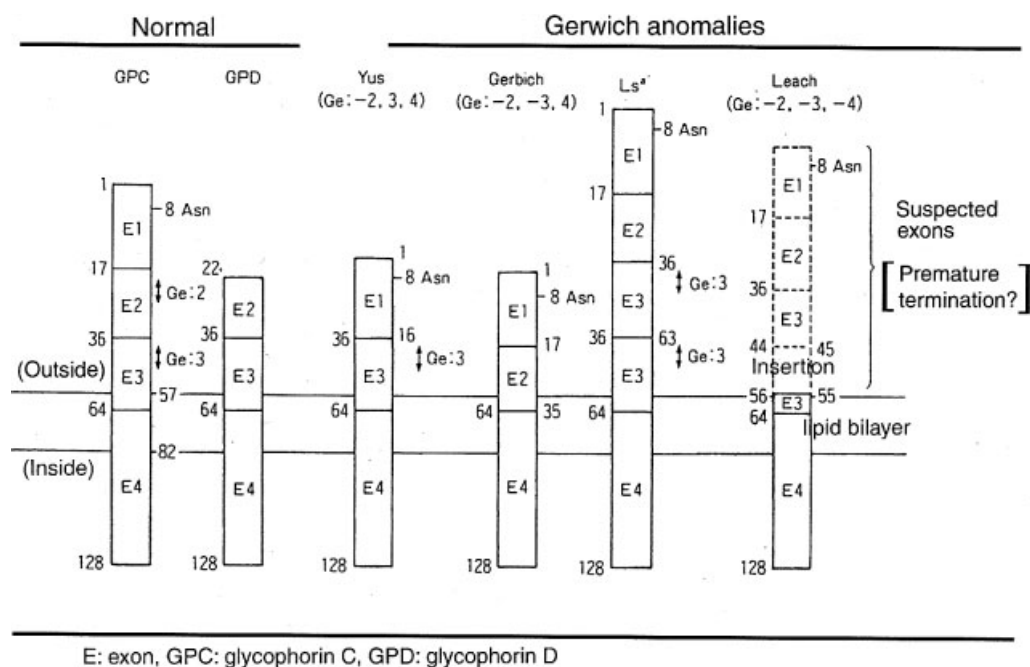


Figure 15.18 Molecular genetics of Gerwich anomalies related to the glycophorins C and D group.

Typical examples of recombination events of the two genes of *GYP A* and *GYP B* are the Miltenberger phenotypes [41] (Fig. 15.15). The Miltenberger V (MiV) phenotype arises as a consequence of recombination of the *GYP A* and *GYP B* genes that have juxtaposed the 5'-segment of the GPA gene with 3'-segment of the GPB gene, with the crossovers occurring within the intron 3' to GPA exon 3 and the intron 5' to GPB exon 4. These hybrid molecules express the antigenic activities of M and S, or M and s, depending on the Ss genotype of the donor GPB segment. A reciprocal cross-over process appears to be responsible for producing the St^a variant glycophorin. The Miltenberger III (Mi III) variant of the glycophorins demonstrates double cross-over events, or gene conversion events, which appear to be responsible for replacing a segment of the GPB gene with a corresponding segment of the GPA gene.

Band 3 knock-out mice demonstrate complete deficiency of GPA in red cells [26, 27].

Although many variants have been reported in GPA and GPB, it should be noted that, even in the complete combined deficiencies of GPA and GPB, there are no abnormalities in red cell morphology, membrane functions, and red cell survival [41, 42]. These observations suggest that the GPA and GPB appear to play no critical role in cellular functions.

15.2.2

Glycophorin C and D Variants

The GPC gene (see Section 5.2.2) is composed of four exons [32, 43]. The extracellular domain of GPC is encoded by exons 1 and 2. Its transmembrane segment is encoded by exons 3 and 4, and the cytosolic portion by exon 4.

Four high-frequency antigens within the Gerwich (Ge or GE) blood group are known as Ge: 1, Ge: 2, Ge: 3, and Ge: 4 (Fig. 15.18). The most common phenotype in normal individuals is Ge: 1, 2, 3, 4. Four variants that lack one or more of these determinants are known [32, 43] as (1) the Melanesian type (Ge: -1, 2, 3, 4) which indicates that this variant lacks the Ge: 1 determinant, (2) the Yussef (Yus) type (Ge: -1, -2, 3, 4), (3) the Gerbich type (Ge: -1, -2, -3, 4), and (4) the Leach type (Ge: -1, -2, -3, -4).

The morphology of the red cells of the Leach phenotype demonstrates elliptocytosis with deficiency of glycophorins C and D [44]. Therefore, the Gerbich antigens correspond to determinants on GPC or GPD. The Ge: 2 determinant, which is sensitive to neuraminidase and trypsin treatments, is present only on GPC. The Ge: 3 determinant, which is destroyed by neuraminidase treatment or trypsin digestion, is expressed on GPC and GPD. The Ge: 2 and Ge: 3 determinants are localized at positions corresponding to exons 2 and 3, respectively.

The molecular pathogenesis of the Leach phenotype (Ge: -1, -2, -3, -4) has been elucidated [45]. Some individuals demonstrate homozygosity of a deletion of *GYP C* at exons 3 and 4, yielding aberrant GPC and GPD molecules defective in the membrane-spanning and cytoplasmic segments [46], although exons 1 and 2 are maintained nearly normally. In other individuals of the Leach phenotype,

there are DNA sequence alterations at a position corresponding to codons 44 and 45 [47], that is, an amino acid substitution of a tryptophan to a leucine at position 44, and additional deletion of a single nucleotide in the adjacent codon. The frame-shift mutation produces a termination codon at a position corresponding to residue 56 of the native GPC. One may expect that the predicted protein of aberrant GPC in this individual would consist of 43 residues from the native sequence followed by 12 new amino acids. This mutated polypeptide would not contain a transmembrane segment of the native molecule.

In the Leach phenotype, elliptocytosis is one of the characteristic features [48]. The deficiency of GPC appears to be not directly responsible for the altered mechanical properties observed in Leach red cells, whereas the mechanical instability appears to be due to a partial deficiency of protein 4.1 associated with aberrant GPC. The instability is fully corrected by introducing protein 4.1 or its spectrin-binding domain into GPC-deficient red cells.

Red cells of the Yussef type (Ge: -1, -2, 3, 4) variant demonstrate a deletion of exon 2 of *GYPC*. As a result of this deletion of exon 2, an aberrant transcript is composed of a 109 amino acid-long protein deficient in the 19 residues encoded by exon 2 [46, 49].

The *GYPC* gene in the Gerbich type (Ge: -1, -2, -3, 4) is abnormal in a deletion of sequences corresponding to exon 3 [46, 49]. This variant gene encodes a shortened 100 amino acid-long GPC molecule deficient in 28 amino acid residues corresponding to exon 3. The aberrant GPC molecule retains a transmembrane and COOH-terminal cytosolic domain but displays truncated forms of the extracellular domain. Variant GPD molecules will be synthesized by these molecular variants through the internal initiation mechanism [32].

The Ls^a phenotype is known as a larger GPC and GPD variant molecule as a consequence of duplicated exon 3 [50, 51]. The Webb (Wb) variant is a trypsin-sensitive antigen produced by an aberrant GPC molecule. The pathogenesis lies on a single base pair sequence difference at codon 8 from an asparagine to a serine, removing the single asparagine-linked glycosylation site on the normal GPC molecule [52].

Other point mutations (the An^a and Dh^a antigens) in the *GYPC* gene have been reported [53, 54].

15.3

Blood Group Antigens

15.3.1

Rh Blood Group Antigens

Abnormalities or polymorphisms of the Rh blood group antigens [32, 55] (see Sections 5.3.2 and 12.4) can be observed on the four occasions as described below: (1) the RhD-negative phenotype, (2) the Rh partial D variants, (3) the polymorphisms of the RhCE antigens, and (4) the rare Rh_{null} and Rh_{mod} phenotypes (Table 5-1).

- (1) The RhD-negative phenotype lacks detectable red cell RhD peptide despite of the presence of normal amounts of the RhCE antigens. This RhD-negative population is about 17% in Caucasians, and only 0.5% in Japanese. These individuals are homozygous for partial or complete deletions of the *RHD* locus [56–58]. There is a rare case with a non-functional *RhD* gene due to a 4 bp deletion in a coding region [59]. In the Japanese population with the RhD-negative phenotype, approximately 27% show the grossly intact *RHD* gene [60].
- (2) The antigenic variants of the RhD polypeptide are also known (D_{II} through D_{VII}) [61]. The phenotype of a given D variant group is defined by the lack of expression of one or more of a series of more than 30 D antigenic epitopes (epD₁ through epD₃₀ and higher) [62, 63]. These variants usually demonstrate various epitopic changes. In most instances, gene conversion events are observed, because the gene conversion appears to be relatively favored by the close physical proximity of the *RHD* and *RHCE* loci, by the marked similarity of nucleotide sequences between the two genes, and by the presence of interspersed repetitive DNA sequence elements within the two loci. Partial D variants are also observed owing to point mutations of the *RHD* and *RHCE* genes themselves [64].
- (3) Polymorphisms of the C/c and E/e phenotypes can be accounted for by amino acid substitutions at residues 103 (C = Ser, c = Pro) and 226 (E = Pro, e = Ala) [65]. The low-frequency antigens C^w and C^x derive from a glutamine to arginine change at codon 41 and from an alanine to threonine change at codon 36, in the *RHCE* locus [66]. The VS antigen corresponds to a leucine to valine substitution at codon 245 in the *RHCE* gene [67]. The weak D (Du) phenotype demonstrates low but detectable levels of D antigen with abnormally low amounts of a qualitatively normal RhD transcript [68]. The RN phenotype shows abnormal RhC and Rhe transcripts that generate hybrid RhCe-D-Ce peptides, which are expressed less efficiently.
- (4) The Rh_{null} phenotype, which is completely deficient in erythroid Rh antigens, demonstrates a chronic, mild to moderate nonimmune hemolytic anemia with stomatocytosis, spherocytosis, and increased osmotic fragility [32, 55, 69]. The Rh_{mod} phenotype red cells show low levels of Rh antigens with a mild compensated hemolytic anemia. Rh_{null} and Rh_{mod} red cells have diminished or absent expression of glycophorin B (the Ss antigens), Duffy determinants, the Rh50 peptide, and the LW glycoprotein [55, 69, 70]. The pathogeneses of the Rh_{null} phenotype lie on homozygosity for silent alleles at the *RH* locus, or on homozygosity for an allele ($X^o r$) at an autosomal locus that is genetically independent of the *RH* locus. Defects in the *RH50* locus is also responsible for the Rh_{null} phenotype owing to frameshift and missense mutations leading to disrupted RH50 protein expression [71]. A splicing abnormality yields an abnormally spliced *RH50* transcript resulting in a non-functional Rh50 polypeptide. Defects in the *RH30* gene itself is naturally pathognomonic for the Rh_{null} or Rh_{mod} phenotypes [72–74].

15.3.2

The Kell Blood Group Antigens (The McLeod Syndrome)

The KEL1 antigen is a powerful immunogenic polypeptide among other human blood group alloantigens, second only to the RhD antigen in its immunogenicity [32, 75] (see Section 5.3.5).

In pathological states of the Kell blood group system, two unusual Kell phenotypes are known.

The first one is known as a K_{null} (or K_o) phenotype, which comes from the absence of the Kell glycoprotein from red cells. All known Kell antigens are deficient in red cells of the homozygous individuals with the K_{null} phenotype. K_{mod} is a genetically heterogeneous phenotype with exceptionally weak Kell antigen reactivity in the red cells. It is interesting to note that K_{null} and K_{mod} red cells demonstrate a normal morphology and normal survival *in vivo* [76, 77]. The exact pathogenesis has not been elucidated [32].

The second one is the McLeod syndrome, which is an X-linked disorder with a defect at a gene of Xk . In the McLeod syndrome, the level of the 93×10^3 Da Kell protein is markedly reduced in the red cells. In addition, an antigen named Kx or KEL15 is partially or totally deficient in the red cells. The most characteristic feature is that these abnormalities are accompanied by acanthocytosis in the red cells with the McLeod syndrome [78]. The red cell survival *in vivo* is clearly shortened. Female carriers with a defective Xk gene have two populations of red cells as a consequence of X chromosome lyonization. One red cell population demonstrates a normal red cell shape with a normal amount of Kell antigens. The other population demonstrates acanthocytosis in the red cells, which are from an erythroid progenitor clone wherein the wild type Xk allele is inactivated.

The Kx (or KEL15) antigen, which is deficient in the McLeod syndrome, is a 37×10^3 Da polypeptide [79]. This protein is a 444 amino acid-long polypeptide with ten membrane-spanning segments [80]. Deficiency of the Kx protein is due to large deletions or splice site mutations on the Xk gene [80–81].

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16

Abnormalities of Anchoring Proteins

16.1

Ankyrin

16.1.1

Introduction

Erythroid ankyrin (Ank1) is a large, 206 kDa, 8.3×10 nm protein that provides the primary linkage between the spectrin–actin–based red cell membrane skeleton and the plasma membrane [1–4] (see Sections 6.1, 10.4). This important cellular localization of membrane proteins may be mediated by the relative affinities of the many different isoforms of ankyrin for target proteins; membrane skeleton proteins, ion transport proteins, and cell-adhesion molecules. The isoform diversity of ankyrin arises from both different gene products and alternative splicing of the same gene product. During erythroid development and maturation, Ank1 is detectable at the stage of colony-forming unit-erythroid (CFU-E) after spectrin synthesis begins, but before band 3 is made (see Chapter 7).

Each spectrin tetramer appears to bind on average only one ankyrin molecule, even though two binding sites are available, probably because ankyrin binding is approximately ten times stronger to spectrin tetramers than to spectrin dimers. Ankyrin also binds to the cytoplasmic domain of band 3 molecules at sites near the N-terminus and near the hinge region with its high affinity (K_d : 10^{-7} to 10^{-8} M). This interaction appears to be critically important, because membrane stability decreases markedly, when the ankyrin–band 3 interaction is selectively disrupted in intact red cells at alkaline pH.

Ankyrin 1, red cell ankyrin, has been identified in erythroid tissue, brain, and muscle. The major form of ankyrin 1 is composed of three domains: (1) an 89 kDa NH₂-terminal membrane domain (amino acids 2 to 827) composed of 24 conserved repeats known as a membrane domain that contains the binding sites for band 3; (2) a 62 kDa spectrin-binding domain (amino acids 828 to 1382) that contains the binding sites for spectrin and vimentin; and (3) a 55 kDa COOH-terminal regulatory domain (amino acids 1383 to 1881). Complex patterns of alternative splicing have been identified in the region encoding the regulatory domain (see Section 6.1.2).

The 89 kDa NH₂-terminal membrane domain (see Section 6.1.2.1) consists of 24 tandem subunits of 33 amino acids (ankyrin repeats). The ankyrin repeats are organized into four subdomains of six repeats each, and these repeats form two distinct but cooperative binding sites for band 3. One site of ankyrin to bind band 3 is on repeats 7 to 12 (subdomain 2), and the other site is on repeat 13 to 24 (subdomains 3 and 4). Ankyrin can bind band 3 with these two sites, which allow band 3 to form a tetramer from a dimeric form in the membrane. Structurally, the ankyrin repeats form a novel L-shaped figure consisting of a β -hairpin followed by two α -helices that pack side-to-side in an antiparallel fashion. Red cell ankyrin exhibits the nonglobular structure.

The 62 kDa spectrin-binding domain (see Section 6.1.2.2) binds to spectrin. The binding site appears to be present at regions in both the beginning and middle of this domain. The principal point of spectrin binding may lie in the highly conserved middle region. The nonconserved site at the region at the beginning of this domain may provide specificity and allow ankyrins to discriminate between spectrin molecules. Regarding the ankyrin–spectrin interaction, spectrin repeat 15 is the complementary binding site for ankyrin on spectrin molecules. As for a role of ankyrin in binding the skeletal network to the membrane, ankyrin and band 3 are the major sites of skeletal attachment in red cells. However, spectrin binding to the membrane can be achieved by some other processes during erythropoiesis (possibly by protein 4.2) even though ankyrin is absent, such as *nb/nb* mice, which totally lack Ank1.

The 55 kDa COOH-terminal regulatory domain (see Section 6.1.2.3) contains many alternative splice sites. At the moment, at least 15 ankyrin variants are known, which differ in size and in function. The best example is ankyrin 2.2, which lacks an acidic 162 amino acid sequence, compared with normal full-sized ankyrin (ankyrin 2.1). Ankyrin 2.2 is thought to be an active form of ankyrin, in which binding to band 3 and spectrin is definitely enhanced. The 162 amino acid repressor sequence binds to ankyrin 2.2 and inhibits its interaction with band 3. Shortened variants of ankyrin (20 to 26 kDa) are observed in muscle cells, which are composed of a unique hydrophobic sequence attached to the C-terminal end of the regulatory domain. The regulatory domain of all ankyrins also contains a region that is homologous to the death domains of proteins involved in apoptosis signaling.

A family of ankyrins (see Section 6.1.4) is now known, each of which is encoded by a different gene. The erythroid type of ankyrin is known as ankyrin 1 or ankyrin_R (gene symbol: *ANK1*) which is present in erythroid cells, muscle, cerebellum, macrophages, and endothelial cells.

The red cell *ankyrin 1* (*ANK1*) gene is located at chromosome 8p11.2, and its size is 160 kb. There are two sizes of messenger RNA (mRNA), 7.2 kb, which is found mainly in mature erythroblasts and reticulocytes, and 9 kb, which is found mainly in immature erythroblasts and neural cells. The red cell ankyrin contains 1881 amino acids that are deduced from its mRNA (7.2 kb) with 42 exons, and there are approximately 1.24×10^5 copies of ankyrin molecules per red cell. A neural protein is known as ankyrin 2 or ankyrin_B (*ANK2*), which is found in

neuronal cell bodies, dendrites, and glial cells. Ankyrin 3 or ankyrin_G (ANK3) is one that is distributed broadly in most epithelia, axons, postsynaptic membranes at the neuromuscular junction, lymphocytes, megakaryocytes, and muscle cells. Although ankyrins 1 and 2 are localized in plasma membranes, isoforms of ankyrin 3, which lack all or part of the membrane domain, are localized in intracellular organelles, especially the Golgi apparatus (ankyrin G₁₁₉) and some lysosomes [5]. There are larger forms of ankyrin, such as the 220 kDa isoforms of ankyrin 2 and ankyrin 3, which have the same three domain structure as erythroid ankyrin 1. The membrane and spectrin domains are highly conserved, but the regulatory domains differ greatly. The 440 kDa isoforms of ankyrin 2 and ankyrin 3 are also known, which are due to the insertion of a 220 kDa rod-like sequence between the spectrin and regulatory domains [6, 7]. This excessively large domain targets these giant ankyrins at regions of neurite outgrowth (in ankyrin 2) or at axon initial segments and the nodes of Ranvier (in ankyrin 3).

A large number of integral membrane proteins other than band 3 also bind to ankyrin, that is: anion exchanger 1 (AE 1), anion exchanger 2 (AE 2), Na⁺/K⁺-ATPase, the electrogenic and amiloride-sensitive Na⁺ channels, the Na⁺/Ca²⁺ exchanger, H⁺/K⁺-ATPase, and adhesive proteins, such as CD44, NrCAM, NgCAM, and neuroglian (see Section 6.1.4).

16.1.2

Ankyrin Mutations in Hereditary Spherocytosis

Abnormalities of ankyrin are the principal cause in the pathogenesis for hereditary spherocytosis, especially in Western countries. No reports have been presented on the essential abnormalities in hereditary elliptocytosis regarding its pathogenesis.

The first observation concerning the role of ankyrin in the pathogenesis of red cell membrane disorders was the identification of the deletion of the short arm of chromosome 8 in patients with hereditary spherocytosis (see Section 10.4.1). Two sisters were identified with a deletion of chromosome bands 8p11.1–p21.2, which was associated with spherocytosis, dysmorphic features, micrognathia, nystagmus, and psychomotor retardation. It was known that the chromosomal localization (*SPH1*) of classical hereditary spherocytosis resided in 8p11, and that hereditary spherocytosis was associated with various abnormalities of chromosome 8, such as its translocations, that is, t (8; 12) (p11; p13), or t (3; 8) (p21; p22), and its deletions, that is, 8p11.1, 8p11.22–8p21.1, or 8p11–p21.1. Some patients with hereditary spherocytosis were associated with a deficiency of red cell glutathione reductase, the chromosome of which is located at 8p21.1. These observations provide evidence for a genetic locus for hereditary spherocytosis in the proximal region of the short arm of chromosome 8. The nature of the hereditary spherocytosis locus was elucidated when it was demonstrated that the gene for ankyrin resides in this region and that these above-mentioned patients lacked the ankyrin gene on the abnormal chromosome. It is now known that the red cell ankyrin gene (*ANK1*) resides at 8p11.2. As discussed above, genetic linkage analyses and cyto-

netic evidence showed that a defect in ankyrin is the primary cause of hereditary spherocytosis in some families.

Twelve different ankyrin mutations in 13 kindreds were first identified from 46 kindreds with both dominant and recessive hereditary spherocytosis [8]. To date, 50 mutations of the ankyrin gene as the pathogenesis of hereditary spherocytosis have been reported worldwide (Table 10.1 and see Section 10.4.1).

This disorder comprises a heterogeneous group of hemolytic anemias characterized by chronic hemolysis with microspherocytosis and increased osmotic fragility and is a consequence of heterogeneous defects in the proteins of the red cell membrane.

By analyses of the red cell membrane proteins using protein biochemistry and a radioimmunoassay, several alternative protein deficiencies have been identified in four distinct subsets of hereditary spherocytosis patients, those with: (1) isolated spectrin deficiency, (2) combined spectrin and ankyrin deficiency, (3) band 3 deficiency, or (4) protein 4.2 deficiency. Corresponding to these categories, gene mutations of ankyrin, band 3, protein 4.2, and β -spectrin have been identified.

Ankyrin deficiency is believed to be a major cause of hereditary spherocytosis, especially of autosomal dominant (AD) transmission in Western countries. An analysis of 166 unrelated patients with hereditary spherocytosis showed a deficiency of ankyrin (and its related spectrins) to be present in 60 % of these unrelated families, band 3 in 23 %, and protein 4.2 in 2 %; 15 % were of unknown origin [9]. It has also been reported that in 80 unrelated patients with hereditary spherocytosis, ankyrin/spectrin deficiency was detected in 55 %, band 3 in 27 %, and protein 4.2 in 3 %; 15 % were of unknown etiology [10]. In addition, ten β -spectrin mutations in 40 families with hereditary spherocytosis associated with spectrin deficiency or combined spectrin and ankyrin deficiencies have been reported [11].

Contrary to these reports in Western countries, biochemical studies of 47 patients of 32 unrelated families of hereditary spherocytosis in the Japanese population indicated the presence of band 3 deficiency in 27 %, protein 4.2 deficiency in 14 %, ankyrin deficiency in 4 %, spectrin deficiency in 8 %, and no abnormality in 47 % when protein contents were evaluated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE), with less than 2 standard deviations, compared with normal control samples [12]. These results imply that ankyrin deficiency occurs less often in the Japanese population, although SDS–PAGE may underestimate the degree of ankyrin deficiency in patients, especially compared with a radioimmunoassay (RIA) or an enzyme-linked immunosorbent assay (ELISA) measurement. In addition, there is a higher frequency of protein 4.2 deficiency in this population.

We further extended our study on the incidence of membrane protein abnormalities in our 60 Japanese patients of hereditary spherocytosis with unrelated families [13]. There were fewer ankyrin deficiencies (7 %), a moderate number of band 3 deficiencies (20 %), and many more protein 4.2 deficiencies (45 %), with 28 % of unknown etiology. In the band 3 gene, 11 mutations pathognomonic for hereditary spherocytosis were identified (three frameshift and eight missense mutations). On the other hand, 2 missense mutations were detected in the *ANK1* gene from 26 unrelated Japanese hereditary spherocytosis (in 52 alleles): (1) ankyrin

Brüggen (CGT→CAT; Arg→His at codon 619 in exon 17), and (2) ankyrin Nara (CTA→CCA; Leu→Pro at codon 1046 in exon 28).

It is well known that the ankyrin content is highly dependent on the extent of reticulocytosis. Therefore, it is not unreasonable to consider the possibility that the deficient ankyrin content, which is expected to be decreased because of the ankyrin gene mutations, could be masked by the presence of marked reticulocytosis associated with increased hemolysis in hereditary spherocytosis. This hypothesis would explain why the clinical severity of hereditary spherocytosis in Japan is usually mild, and why the mildly decreased ankyrin content can easily be compensated for by the increased reticulocytosis. Therefore, we extended our studies on the detection of ankyrin gene mutations to a larger scale [14].

As a result, 16 of 49 patients were found to carry ankyrin gene mutations in heterozygous states (Table 16.1). The mutations pathognomonic for hereditary spherocytosis consisted of four nonsense mutations, eight frameshift mutations, and four abnormal splicing mutations (33 % of total unrelated hereditary spherocytosis patients). These mutations are located throughout the ankyrin peptide, and all except one (ankyrin Kagoshima or ankyrin Yamanashi) of these are family-specific, private mutations. It is interesting to note that only four mutations (one frameshift, one abnormal splicing, and two nonsense mutations) were detected in 16 unrelated patients of autosomal dominantly inherited hereditary spherocytosis patients and that 12 mutations (seven frameshift, three abnormal splicing, and two nonsense mutations) were found in 30 sporadic (*de novo*) hereditary spherocytosis patients which were genetically proven because the parents did not have the mutation, contrary to the previous commonly-held belief that null mutations (frameshift and nonsense mutations) predominate in dominant hereditary spherocytosis and that missense mutations tended to be detected mostly in patients of non-autosomal dominant transmission. In clinical hematology [14], the patients with these ankyrin gene mutations tended to be slightly more anemic ($p < 0.002$) and to have a higher level of reticulocytosis ($p < 0.011$) than those with the band 3 gene mutations [13, 14]; the levels of hemoglobin, mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), reticulocytes, and indirect bilirubin were $9.0 \pm 2.3 \text{ g dL}^{-1}$, $87.0 \pm 6.6 \text{ fL}$, $33.3 \pm 2.2 \%$, $15.8 \pm 7.4 \%$, and $2.1 \pm 1.6 \text{ mg dL}^{-1}$, respectively, in the group with ankyrin gene mutations, compared with $12.7 \pm 1.7 \text{ g dL}^{-1}$, $88.1 \pm 6.0 \text{ fL}$, $36.4 \pm 1.2 \%$, $8.4 \pm 1.2 \%$, and $2.1 \pm 1.4 \text{ mg dL}^{-1}$, respectively in the group with band 3 gene mutations.

In this study, in addition to the above-mentioned two missense mutations (ankyrin Brüggen, and ankyrin Nara), 15 silent mutations were also detected [13, 14] (Table 16.2). Regarding the allele frequency of these ankyrin gene polymorphisms in the Japanese population, 2997C→G was the most frequent (0.49 in 49 hereditary spherocytosis patients and 0.52 in 46 healthy subjects), followed by 3044 (+46C→T) (0.23 and 0.30, respectively), 681G→A (0.18 and 0.16, respectively), and 399C→T (0.14 and 0.15, respectively) [13, 14]. No significant difference was observed in the allele frequency of these gene polymorphisms between normal subjects and hereditary spherocytosis patients. These polymorphisms were observed to almost the same extent in Japanese and German populations [13, 14].

Table 16.1 Genotypic and phenotypic characteristics in Japanese HS probands with ankyrin gene mutations.

Name of mutation	Location	Nucleotide	Type of mutation	Inheritance	Hb (g dL ⁻¹)	Retic (%)	Spectrin Ankyrin P4.2 Band 2.2 Splenectomized			
							(% of normal)			
Ankyrin Chiba II	Exon 1, codon 2–5	88–97nt <u>CCCTAATCTG</u> →TG	Frameshift (PCT)	AD (heterozygous)	5.4	7.3	101	92	72	70
Ankyrin Nara	Intron 1, 5nt after end of exon 1	AAgtgag→Agtgac	Abnormal splicing?	AD (heterozygous)	10.9	19.8	91	105	97	90
Ankyrin Saitama	Exon 5, codon 111 or 112	419–420nt GGTTT→GGTTT	Frameshift (PCT)	non-AD (heterozygous)	15.1	1.8	91	102	98	96
Ankyrin Shiga	Intron 5, 3–4nt after end of exon 5	AAgtaag→AAgtaaag	Abnormal splicing?	non-AD (heterozygous)	10.0	21.4	91	107	88	76
Ankyrin Tokyo II	Exon 6, codon 187–190	645–654nt CGCACGGCTGCG→CG	Frameshift (PCT)	de novo (heterozygous)	11.6	5.6	101	93	76	79
Ankyrin Kyoto*	Intron 8, 5nt after end of exon 8	AGgtggg→AGgtggc	Abnormal splicing?	AD (heterozygous)	9.3	14.4	93	113	86	103
Ankyrin Tokyo III	Exon 16, codon 571, 572 or 573	ACCCCCCTG→ACCCCCCTG	Frameshift (PCT)	de novo (heterozygous)	7.3	16.8	93	112	87	96
Ankyrin Aichi*	Exon 16, codon 592–593	1860–1861nt GGCGGC→GGCGGGCGGC	Frameshift (PCT)	de novo (heterozygous)	9.7	6.0	101	91	82	86
Ankyrin Osaka II	Exon 17, codon 612	1918nt CAG→TAG	Nonsense	de novo (heterozygous)	9.2	8.4	100	100	89	86
Ankyrin Osaka I	Exon 17, codon 637	1994–1995nt ACG→ACCG	Frameshift (PCT)	non-AD (heterozygous)	9.2	23.8	106	92	78	64
Ankyrin Kagoshima	Exon 22, codon 798–799	2478–2481nt GTCAGT→GT	Frameshift (PCT)	non-AD (heterozygous)	9.7	20.6	96	96	90	83

Table 16.1 Continued

Ankyrin Yamanashi	Exon 22, codon 798–799	2478–2481nt <u>GTCAGT</u> → <u>GT</u>	Frameshift (PCT)	de novo (heterozygous)	8.1	21.8	101	116	78	85	–
Ankyrin Yamagata	Intron 22, 1nt after end of exon 22	AG <u>g</u> t→AG <u>c</u> t	Abnormal splicing?	non-AD (heterozygous)	6.6	9.2	103	107	96	107	–
Ankyrin Mie	Exon 26, codon 951–953	2936–2942nt <u>TGCGGCGCTG</u> → <u>TTCGGG</u> and + <u>TCTG</u>	Frameshift	de novo (heterozygous)	10.6	11.4	100	107	83	110	–
Ankyrin Chiba I	Intron 28, 1nt after end of exon 28	AG <u>g</u> t→AG <u>c</u> t	Abnormal splicing?	non-AD (heterozygous)	6.9	21.5	97	122	77	69	–
Ankyrin Chiba III	Exon 31, codon 1230	3774nt <u>TAC</u> → <u>IAG</u>	Nonsense	non-AD (heterozygous)	9.2	22.4	96	109	84	56	–
Ankyrin Tokyo I	Exon 31, codon 1252	3838nt <u>CGA</u> → <u>TGA</u>	Nonsense	AD (heterozygous)	7.9	24.8	100	115	89	84	–
Ankyrin Chiba IV*	Exon 36, codon 1437	4393nt <u>GTG</u> → <u>TG</u>	Frameshift (PCT)	non-AD (heterozygous)	10	11	109	108	79	84	–
Ankyrin Okayama*	Intron 36, 1nt be- fore start of exon 37	ag <u>TG</u> →aa <u>TG</u>	Abnormal splicing?	AD (heterozygous)	13.4	7.7	104	92	83	78	–
Ankyrin Toyama	Exon 38, codon 1640	5002nt <u>CAG</u> → <u>TAG</u>	Nonsense	AD (heterozygous)	11	12.8	98	99	88	154	–

nt indicates nucleotide; PCT, premature chain termination; AD, autosomal dominant.

* These mutations have recently been identified as new mutations.

Table 16.2 Polymorphism and allele frequency of the ankyrin-1 gene in healthy control subjects and HS probands.

Location	Polymorphism	Name of variant	Allele frequency	
			HS (n = 62)	Normal (n = 46)
Intron 1	C→T, 84nts before start of exon 2	122 (−84C→T)	0.00	0.01
Exon 2	codon 11: <u>G</u> <u>A</u> <u>T</u> → <u>G</u> <u>C</u> <u>T</u> (Asp→Ala)	D11A	0.01	0.01
Exon 4	codon 105: <u>A</u> <u>A</u> <u>C</u> → <u>A</u> <u>A</u> <u>T</u> (silent)	399C→T	0.12	0.15
Exon 6	codon 199: <u>C</u> <u>C</u> <u>G</u> → <u>C</u> <u>C</u> <u>A</u> (silent)	681G→A	0.18	0.16
Intron 7	C→T, 32nts before start of exon 8	796 (−32C→T)	0.01	0.02
Exon 17	codon 619: <u>C</u> <u>G</u> <u>T</u> → <u>C</u> <u>A</u> <u>T</u> (Arg→His)	R619H	0.05	0.02
Exon 18	codon 691: <u>G</u> <u>G</u> <u>C</u> → <u>G</u> <u>G</u> <u>T</u> (silent)	2157C→T	0.12	0.10
Exon 20	codon 737: <u>C</u> <u>C</u> <u>C</u> → <u>C</u> <u>C</u> <u>G</u> (silent)	2295C→G	0.01	0.01
Exon 21	codon 783: <u>A</u> <u>C</u> <u>C</u> → <u>A</u> <u>C</u> <u>T</u> (silent)	2433C→T	0.12	0.10
Intron 22	T→C, 13nts after end of exon 22	2545 (+13T→C)	0.01	0.00
Exon 26	codon 971: <u>C</u> <u>T</u> <u>C</u> → <u>C</u> <u>T</u> <u>G</u> (silent)	2997C→G	0.45	0.52
Intron 26	C→T, 46nts after end of exon 26	3044 (+46C→T)	0.23	0.30
Intron 28	C→G, 21nts after end of exon 28	3411 (+21C→G)	0.06	0.04
Exon 33	codon 1367: <u>G</u> <u>C</u> <u>C</u> → <u>G</u> <u>C</u> <u>T</u> (silent)	4185C→T	0.05	0.05
Exon 39	codon 1755: <u>G</u> <u>T</u> <u>G</u> → <u>G</u> <u>T</u> <u>A</u> (silent)	5439G→A	0.15	0.21
Intron 40*	C→T, 3nts before start of exon 41	5563 (−3C→T)	0.81	0.77
Intron 41	G→A, 71nts after end of exon 41	5703 (+71G→A)	0.15	0.17
Intron 41	C→T, 364nts after end of exon 41	5703 (+364C→T)	0.01	0.00

nt indicates nucleotide, HS: hereditary spherocytosis.

* This polymorphism has recently been detected as a new one.

Up to the present time, more than 50 ankyrin gene mutations have been reported in autosomal dominantly inherited hereditary spherocytosis (Tables 10.1 and 16.1). They are spread over the ankyrin gene (Fig. 1.5), and most of them are private mutations with a few exceptions such as of ankyrin Kagoshima (or ankyrin Yamanashi), or of ankyrin Florianopolis, which is a recurrent frameshift mutation with severe dominantly inherited hereditary spherocytosis. *De novo* mutations in one of the ankyrin alleles leading to decreased expression are frequent in patients with hereditary spherocytosis without a positive family history.

In most of the patients with the ankyrin gene mutations, one ankyrin allele has reduced expression in a third of the hereditary spherocytosis patients with combined spectrin and ankyrin deficiency [1–4]. This implies that transcription of one of the ankyrin alleles is either reduced or its mRNA is unstable. In fact, most of the null mutations are not detectable in reticulocyte mRNA, suggesting instability of the mutant transcript.

In clinical phenotypes of these patients with the ankyrin gene mutations, the extent of anemia is variable, and ranges from mild to severe, probably because the null mutation appears to be compensated to different degrees, such as the presence of marked reticulocytosis either with overproduction of the normal ankyrin allele or with diminished ankyrin degradation [15–18]. This mechanism is still puzzling.

All of the ankyrin gene mutations are accompanied by combined and equivalent deficiencies of ankyrin and spectrin. Historically, the first report to be presented indicated that spectrin is deficient in patients with hereditary spherocytosis [19]. The extent of spectrin deficiency correlates well with the clinical severity, such as the degree of anemia, of spherocytosis, and of increased osmotic fragility. In addition, the spectrin deficiency in hereditary spherocytosis patients became known as usually being secondary to or associated with other skeletal protein deficiencies, especially with ankyrin. Ankyrin represents the principal binding site for spectrin on the membrane (see Sections 4.1 and 6.1). Thus, it is not surprising that ankyrin deficiency is associated with a proportional decrease in spectrin assembly on the membrane in spite of a normal synthesis of spectrin. Through detailed studies, it has been shown that 30 to 45 % of hereditary spherocytosis patients have combined ankyrin and spectrin deficiency, and about 30 % have isolated spectrin deficiency [9, 10].

Among the ankyrin gene mutations reported, several interesting mutations are known. Ankyrin Rakovnik [20], which is a nonsense mutation (E1669X) within the regulatory domain of the ankyrin gene in a patient with autosomal dominantly inherited hereditary spherocytosis, preserves band 2.2 as the minor isoform, in spite of selective deficiency of band 2.1 as the major ankyrin isoform. Ankyrin Walsrode [8], which is a missense mutation (V463I) within the band 3 binding membrane domain in a patient of recessive hereditary spherocytosis, exhibits a decreased affinity for band 3, resulting in a marked deficiency of band 3, more than that of ankyrin or spectrin.

Regarding animal models, six types of autosomal recessively inherited hereditary hemolytic anemia are known in the common house mouse (*Mus musculus*), such as *ja/ja*, *sph/sph*, *sph^{ha}/sph^{ha}*, *sph^{2BC}/sph^{2BC}*, *sph^{1J}/sph^{1J}*, *sph^{2J}/sph^{2J}*, *sph-Dem/sph-Dem*,

and *nb/nb* [21]. All of the homozygous mutations exhibit severe hemolysis with striking reticulocytosis, marked spherocytosis, icterus, biliary gallstones, and massive hepato-splenomegaly. A mild clinical phenotype with a mild spectrin deficiency is shown by an *sp/sp* mutant in the deer mouse (*Peromyscus maniculatus*).

Among these mouse mutants, the *nb/nb* mice exhibit a total lack of ankyrin with concomitant reduction of the spectrin content (50 to 70 % of normal values) [22]. In these mice, synthesis of spectrin is basically unaffected [23]. The major pathogenesis of spectrin deficiency appears to be due to extreme instability of the ankyrin molecules. It is interesting to note that the *nb/nb* mice develop ataxia due to loss of cerebellar Purkinje cells when they are grown up [24]. In the Purkinje cells of the mice, Ank1 protein is markedly decreased [25–27].

No ankyrin mutations have been reported in hereditary elliptocytosis.

16.1.3

Ankyrin Marburg and Ankyrin Stuttgart

Ankyrin mutations are one of the major pathogenesis for hereditary spherocytosis. These mutations are observed typically in the heterozygous state of this disease of autosomal dominant inheritance. Null mutations (frameshift and nonsense mutations) that result in either unstable ankyrin transcripts or truncated peptides predominate in dominant hereditary spherocytosis. These ankyrin defects lead to combined and equivalent deficiencies of ankyrin and spectrin. Most of the null mutations are not detectable in reticulocyte mRNA, probably due to instability of the mutant transcript. The clinical phenotypes exhibit a marked variability that depends on different degrees of compensation for the null mutation, either from overproduction of normal ankyrin or diminished ankyrin degradation.

The exact abnormalities of red cell membranes *in situ* in these disorders with ankyrin defects have never been studied in detail. Therefore, patients with two ankyrin mutations in autosomal dominantly inherited hereditary spherocytosis were selected to study red cell membranes *per se* by electron microscopy [28]. The first one is ankyrin Stuttgart [8] with a frameshift mutation on the ankyrin gene, in which two nucleotides are deleted at nucleotides 985 and 986 in codon 329. The second one is ankyrin Marburg [8] with a frameshift mutation on the ankyrin gene, in which four nucleotides are deleted at nucleotides 2389–2392 in codon 797 (Table 10.1 and Fig. 16.1). Both mutations are located at the band 3 binding domain at the N-terminal region of the red cell ankyrin 1 gene.

In the proband with ankyrin Marburg, the hemoglobin level was 7.8 g dL⁻¹ with increased reticulocytosis (7.4 %), increased mean cell hemoglobin concentration (MCHC: 38.8 %), and marked microspherocytosis (Fig. 16.2) indicating the presence of moderate to severe hemolytic anemia before splenectomy. Under this unsplenectomized condition, the contents of ankyrin, spectrin, and band 3 were –55 %, –38 %, and –22 %, compared with those in normal subjects, when they were determined by radioimmunoassay.

The cytoskeletal network of the red cell membranes of the patients with ankyrin Marburg was examined by electron microscopy with the quick-freeze deep-etching

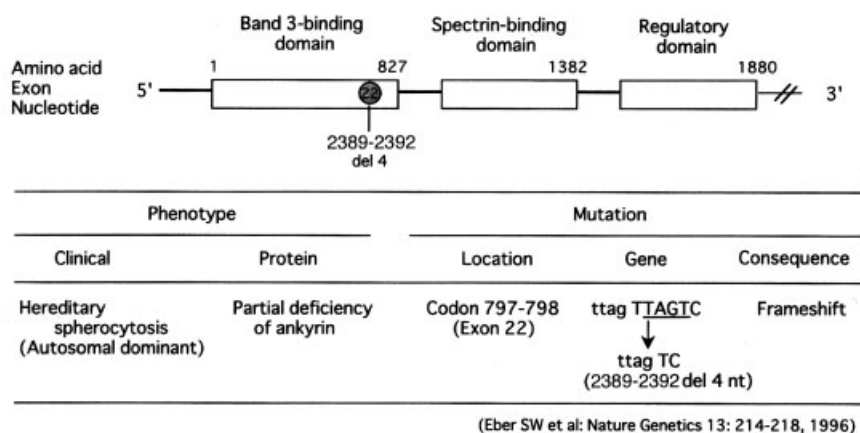


Figure 16.1 A frameshift mutation of the human erythroid ankyrin gene (ankyrin Marburg) in autosomal dominantly inherited hereditary spherocytosis.

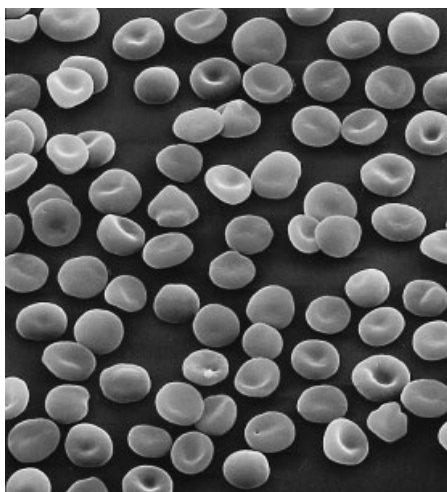


Figure 16.2 Scanning electron micrograph of peripheral red cells in ankyrin Marburg with microspherocytosis.

method [28] (Fig. 16.3). The number of cytoskeletal units per μm^2 was 548 ± 39 in normal subjects, and 386 ± 20 ($70.4 \pm 3.6\%$ of the normal) in the mother (proband), 415 ± 16 ($75.7 \pm 2.9\%$) and 393 ± 37 ($71.7 \pm 6.8\%$) in her two affected daughters. Therefore, the decrement of cytoskeletal proteins, especially of spectrin and ankyrin was also quantitatively confirmed by electron microscopy. In these cytoskeletal networks, the small sized (20–44 nm in diameter) cytoskeletal units in the mother were reduced from $70 \pm 10\%$ that of normal subjects to $46 \pm 5\%$, concomitant with increased medium sized (45–68 nm), and large sized (69–92 nm) in the mother's red cells, suggesting that the cytoskeletal units are partly disrupted due to the decreased ankyrin and spectrin contents. The intramembrane particles

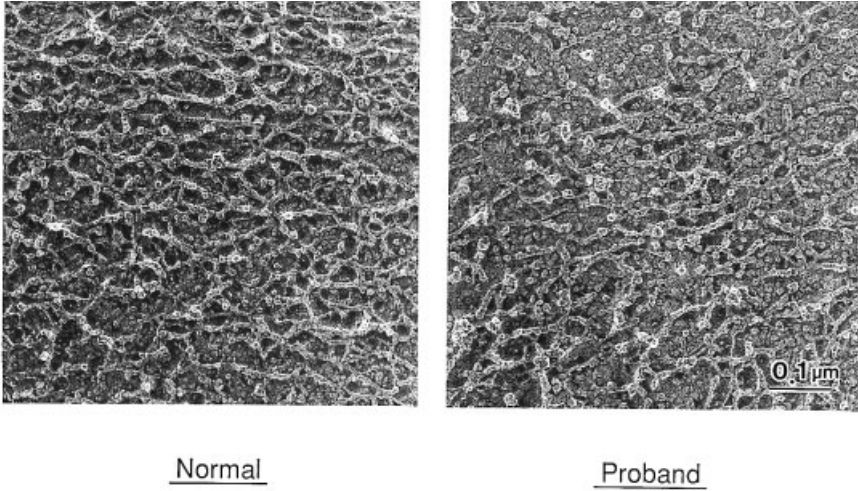


Figure 16.3 Disrupted cytoskeletal network in red cell membranes of hereditary spherocytosis with the frameshift mutation (ankyrin Marburg) examined by electron microscopy with the quick-freeze deep-etching method.

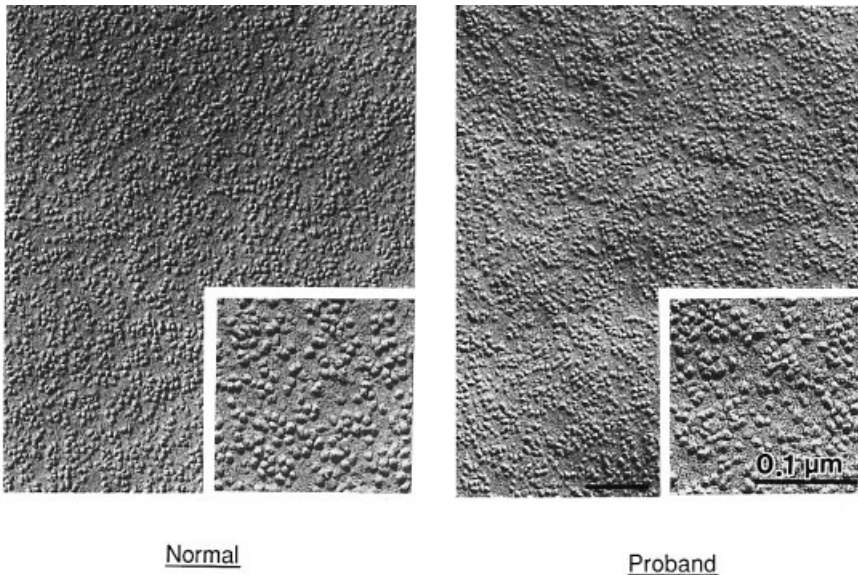


Figure 16.4 Intramembrane particles in red cell membranes of hereditary spherocytosis with a heterozygous frameshift mutation (ankyrin Marburg) examined by electron microscopy with the freeze fracture method.

Ankyrin

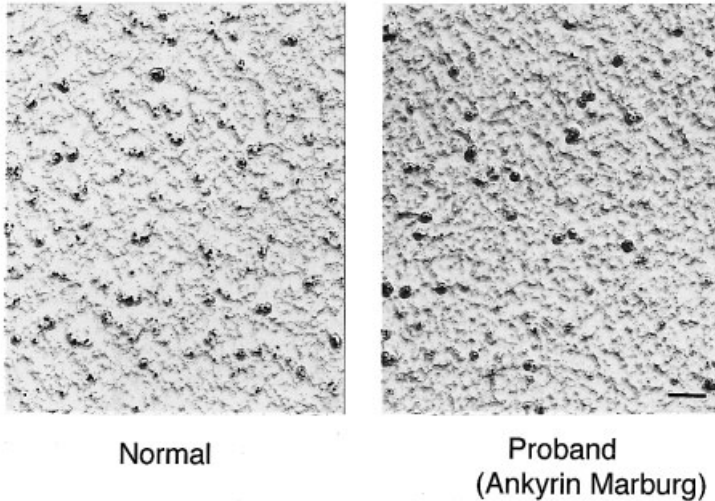


Figure 16.5 Immuno-electron micrograph of red cell membranes in the ankyrin gene mutation (ankyrin Marburg) examined by the surface replica method by utilizing the anti-human ankyrin antibody.

(IMPs), most of which are thought to be band 3 molecules, were also examined by electron microscopy with the freeze fracture method (Fig. 16.4). The number of IMPs per μm^2 was 5367 ± 220 in normal subjects and 5442 ± 241 in the mother, indicating that the state of band 3 was essentially not affected. The localization of ankyrin molecules on the red cell membranes *in situ* was markedly deranged [28], indicating that the availability of epitopes of ankyrin molecules against the anti-ankyrin antibody was extremely low compared with the protein content of ankyrin, when examined by immun-electron microscopy with anti-ankyrin antibody (Fig. 16.5). This result strongly suggests that conformational change in ankyrin molecules is present with the frameshift mutations in ankyrin Marburg, the same as in ankyrin Stuttgart.

16.2

Protein 4.2

16.2.1

Introduction

Protein 4.2 abnormalities of congenital origin (see Sections 6.2 and 10.4) are classified into two groups, i.e.: (1) deficiency (reduced content) of protein 4.2 (Fig. 16.6), and (2) protein 4.2 variants with normal or nearly normal protein 4.2 content [29–32] (Table 16.3).

Table 16.3 Genetic and phenotypic characteristics in HS patients with protein 4.2 gene mutations.

Exon	Nucleotide changes	Amino acid changes	Type of mutations	Inheritance	Red cell morphology	Protein 4.2 content (iso-forms in kDa)	Race	Numbers of kindred	Numbers of patients	Name of mutants	Reference
2	264 or 265 AAG GTG→AAGTGG	88KV→KW	Frameshift	AR (homozygote)	HS	None	P	1	1	P4.2 Lisboa	[39]
3	357 TGG→TGA	119W→Stop	Nonsense	AR (compound heterozygote with P4.2 Nippon)	HS	Trace (74/72)	J	1	2	P4.2 Fukuoka	[36]
3	424 GCT→ACT	A142T	Missense	AR (homozygote)	OS	Trace (74/72)	J	13	17	P4.2 Nippon*	[33, 34, 35]
4	523 GAT→TAT	D175Y	Missense	AR (homozygote)	OS	None	J	1	1	P4.2 Komatsu*	[38]
6	The first base of intron 6: G→A	Exon 6 spliced	Splicing	AR (compound heterozygote with P4.2 Nippon)	HS	Trace (74/72)	J	1	1	P4.2 Notame	[41]
7	929 CGA→CAA	R310Q	Missense	AR (homozygote)	HS	None	T	1	2	P4.2 Tozeur	[40]
7	949 CGC→TGC	R317C	Missense	AR (compound heterozygote with P4.2 Nippon)	MS	Trace (74/72)	J	1	2	P4.2 Shiga*	[37]
7	950 CGC→CC	PCT	Frameshift	AR (homozygote)	H	None		1	1	P4.2 Nancy	[42]

Abbreviations: P4.2, protein 4.2; AR, autosomal recessive inheritance; P, Portuguese; J, Japanese; T, Tunisian; OS, ovalostomatocytosis; MS, microspherocytosis; HS, hereditary spherocytosis.

* Cases studied at the Kawasaki Medical School.

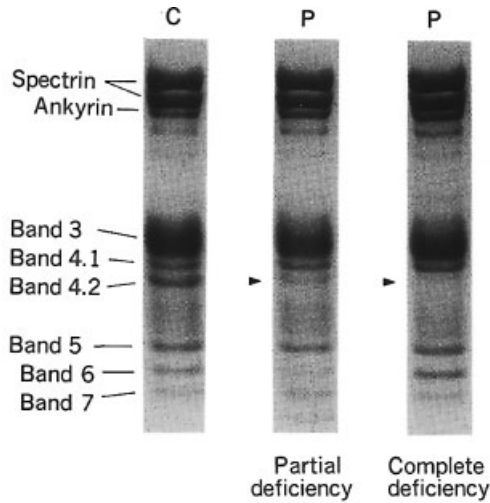


Figure 16.6 A partial or complete deficiency of protein 4.2 in red cells on the sodium dodecylsulfate polyacrylamide gel electrophoresis. C: Control, P: patient.

Membrane proteins were separated by the Fairbanks' nonlinear 3.5–17% polyacrylamide gradient SDS slab gel electrophoresis.

The first category of protein 4.2 deficiencies is further divided into two subgroups: i.e.: (1) total deficiency, and (2) partial deficiency.

16.2.2

Total Deficiency of Protein 4.2

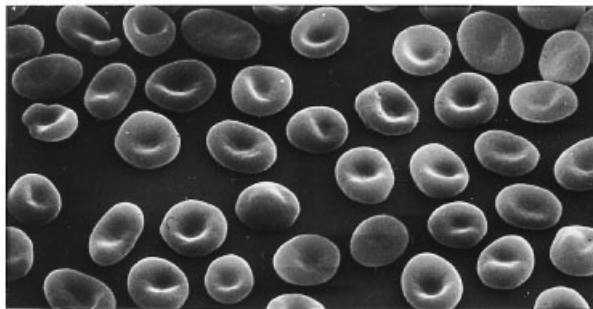
16.2.2.1 Clinical Hematology

Although a slight variation in clinical observations exists among the reported cases with total protein 4.2 deficiency [33–42], the characteristic clinical features are moderate, uncompensated hemolysis with moderate reticulocytosis, and increased indirect bilirubin. The hemolysis usually responds to splenectomy. Regarding cell hydration, the mean corpuscular hemoglobin concentration (MCHC) in protein 4.2 deficiency is minimally elevated, in comparison with typical cases of classical, autosomal-dominantly inherited HS, in which substantial microspherocytosis and increased MCHC are typical. In 17 cases with protein 4.2 Nippon [35], who were homozygotes of a missense mutation (codon 142 GCT→ACT) of the protein 4.2 gene, red cell counts were $3.78 \pm 0.41 \times 10^6 \mu\text{L}^{-1}$, MCHC $34.8 \pm 0.1 \text{ g dL}^{-1}$, and reticulocytes $6.2 \pm 2.5 \%$. A homozygote with protein 4.2 Komatsu [38], who carried a missense mutation (codon 175 GAT→TAT) of the protein 4.2 gene, demonstrated lower red cell counts ($3.3 \times 10^6 \mu\text{L}^{-1}$), and higher MCHC (35.2 g dL^{-1}) and reticulocytes (12.4%). In a compound heterozygote with protein 4.2 Nippon and protein 4.2 Shiga [37] of a missense mutation (codon 317 CGC→TGC) of the protein 4.2 gene, red cell counts were $4.36 \times 10^6 \mu\text{L}^{-1}$, MCHC 37.4 g dL^{-1} , and reticulocytes 4.0%.

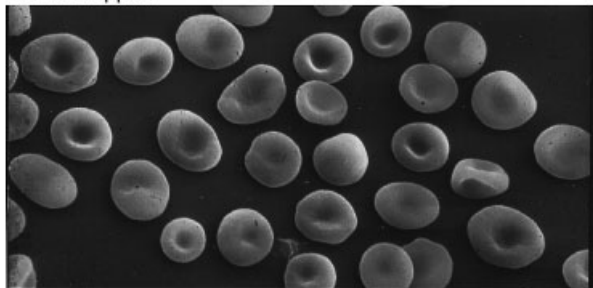
Red cell morphology varies among the patients with protein 4.2 deficiency (Fig. 16.7). Protein 4.2 Nippon [35] and protein 4.2 Komatsu [38] patients consistently demonstrate ovalostomatocytosis, which is characterized by the presence of elliptic cells (not exceeding 20 %) and stomatocytic changes superimposed on both discoid and elliptocytic red cells. Microspherocytosis is only minimal. Instead, red cell morphology in protein 4.2 Shiga [37] is characterized by microspherocytosis, which can barely be differentiated from that of classical hereditary spherocytosis (HS). Other patients with protein 4.2 deficiency have been reported as suffering from HS or the like in their red cell morphology.

The indirect bilirubin level was $1.8 \pm 0.9 \text{ mg dL}^{-1}$ in the reported patients. The osmotic fragility of the patients' red cells was consistently increased.

A. P.4.2 Komatsu



B. P.4.2 Nippon



C. P.4.2 Shiga

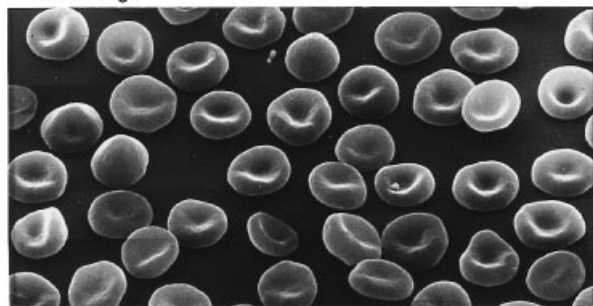


Figure 16.7 Red cell morphology in the peripheral blood of patients with total protein 4.2 deficiency examined by scanning electron microscopy. A: Protein 4.2 Komatsu, B: protein 4.2 Nippon, and C: protein 4.2 Shiga.

16.2.2.2 Red Cell Membrane Proteins

Protein 4.2 content was completely or nearly completely missing in the red cells of these patients (Fig. 16.8), when studied by SDS-PAGE with Coomassie blue staining and also by the Western blot analysis with anti-protein 4.2 antibody. In the Nippon type [35], trace amounts of the 72 and 74 kD peptides were detected by immunoblotting, when an excess amount (50 μ g) of the ghost proteins was applied. The amount of the 74 kDa peptide was nearly identical to that of the 72 kDa peptide. This 74 kDa peptide was also detected at a trace level by immunoblotting even in asymptomatic heterozygous Japanese individuals who have the 142 GCT→ACT point mutation. The incidence of the 74 kDa peptide in a trace amount detected in normal Japanese subjects was approximately 3%. The 74 kDa peptide was not detected in protein 4.2 Komatsu without the missense mutation of codon 142 GCT→ACT. In contrast, it was present among the compound heterozygous patients with protein 4.2 Shiga and protein 4.2 Nippon.

In the protein 4.2 deficiency, the content of band 3 was reduced by 10~20% that of the normal value. The contents of most other membrane proteins, including spectrin, ankyrin, and protein 4.1 were essentially unaffected.

It has recently been shown that protein 4.2-deficient red cells lack CD 47 implicating an interaction between the Rh complex and the band 3 complex [82].

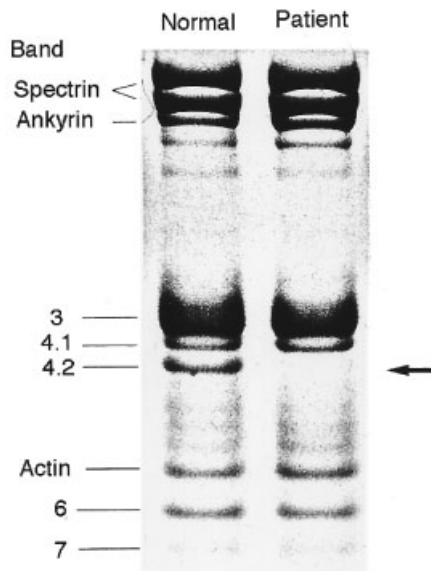


Figure 16.8 Membrane protein profiles of red cells of patients with complete protein 4.2 deficiency examined by sodium dodecylsulfate polyacrylamide gel electrophoresis.

(Arrow indicates deficiency of protein 4.2)

16.2.2.3 Red Cell Membrane Lipids

The content of red cell membrane lipids (see Section 2.2) appeared to be normal in relation to total lipid content, free cholesterol, total phospholipids and their sub-fractions in the seven unsplenectomized patients with protein 4.2 deficiency, when studied by standard methods with thin-layer chromatography.

In protein 4.2 deficiency [30, 31, 35] ($n = 7$), red cell free cholesterol (FC) content was $1204 \pm 59 \mu\text{g}$ per 10^{10} red cells, compared with 1202 ± 103 in normal controls ($n = 152$) and 1084 ± 63 in unsplenectomized HS ($n = 15$). The total phospholipid content was 2548 ± 60 in protein 4.2 deficiency, 2604 ± 241 in normal controls, and 2302 ± 169 in unsplenectomized HS; phosphatidylethanolamine (PE) 734 ± 48 , 802 ± 79 , and 668 ± 53 ; phosphatidylserine (PS) and phosphatidylinositol (PI) 436 ± 30 , 367 ± 36 , and 349 ± 21 ; phosphatidylcholine (PC) 688 ± 51 , 729 ± 60 , and 664 ± 56 ; sphingomyelin (SM) 645 ± 20 , 663 ± 67 , and 589 ± 45 ; lysophosphatidylcholine (L-PC) 47 ± 15 , 39 ± 11 , and 32 ± 7 , respectively.

16.2.2.4 Red Cell Deformability

The membrane deformability of protein 4.2 deficient red cells has been reported as normal when studied in a non-stressed condition by ektacytometry [30, 31, 33, 35]. Under conditions with various stresses such as heat treatment, however, membrane deformability is distinctly abnormal [30, 31] (Fig. 16.9). Freshly drawn intact red cells from protein 4.2 deficient patients were studied by ektacytometry. The red cell suspensions were subjected to heat treatment from 37°C up to 48°C , and examined under various shear stresses ($0\text{--}3000 \text{ dyn cm}^{-2}$). Red cell deformability was expressed as a deformability index. Marked impairment of red cells deformability was observed in the protein 4.2 deficiency especially beyond 46°C . The deformability index in protein 4.2 deficient red cells was markedly decreased to 88 % of that of normal controls at 45°C , 80 % at 46°C , 61 % at 47°C , and 52 % at 48°C . The extent of the abnormality in the protein 4.2 deficiency was strikingly different from that in classical HS, in which no essential changes were observed under the same conditions, as was the case with normal controls [30, 31]. This finding indicates that protein 4.2 deficient red cells with the 142 GCT $\rightarrow$ ACT point mutation possess characteristic physiochemical properties different from those of red cells of classical HS with normal protein 4.2 content. Under heat treatment up to 48°C , no marked changes in red cell shape (budding formation and other poikilocytic shapes) were observed in the deficient red cells. The MCHC was also unchanged. The contribution of spectrins to the marked impairment of the cytoskeleton was evaluated by incubating 37°C extracted crude spectrin at various temperatures (0 , 46 or 48°C) to study the dimer-to-tetramer conversion (see Sections 4.1 and 11.3.2). The association constants (K_a) of dimer-to-tetramer conversions and the contents of high molecular weight (HMW) spectrin were essentially normal in these deficient red cells. In another experiment, tetramer-to-dimer conversion of spectrin was performed in the ghost membranes of red cells, which were subjected to 4 , 46 or 48°C for 10 min. Spectrin was extracted at 4°C , and the contents of dimer and of HMW were calculated yielding normal results in these deficient red cells.

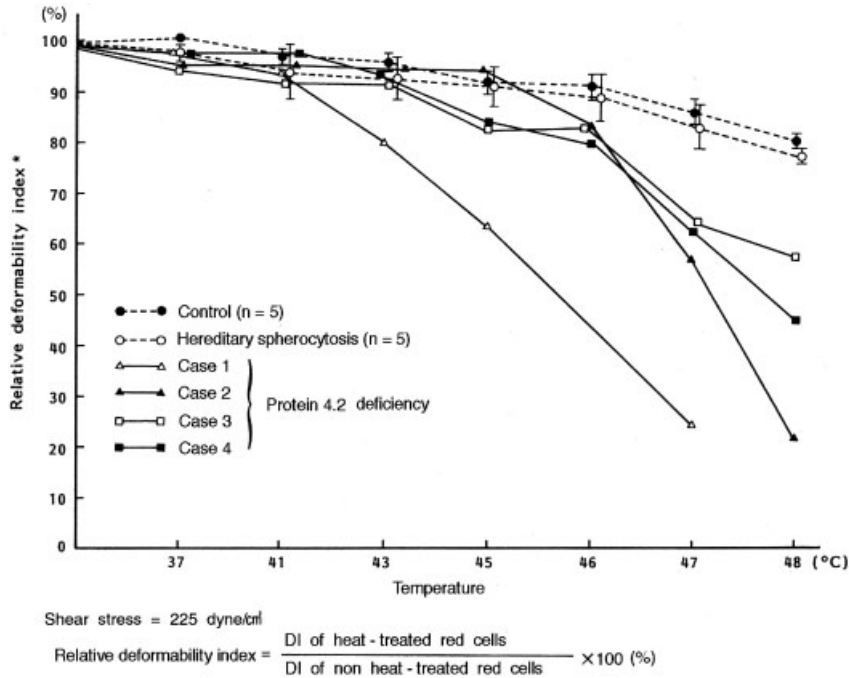


Figure 16.9 Significantly decreased membrane deformability of protein 4.2 deficient red cells under heat treatment examined by ektacytometry.

Furthermore, spectrin was extracted at 4 °C from red cell ghosts prepared from protein 4.2 deficient red cells treated with heat (4, 46 or 48 °C). The contents of dimer and of HMW were normal. These results indicate that spectrin function itself appears to be nearly normal over a wide range of temperatures.

Red cell deformability is chiefly dependent on the functions of the cytoskeletons (see Section 2.3.4.2), which are composed predominantly of spectrin in addition to protein 4.1 and actin. Therefore, if cytoskeletal functions are disrupted by the absence of protein 4.2, the red cells should lose their normal integrity in cell deformability. This was found to be so with protein 4.2 deficiency after heat treatment up to 48 °C; the patient's red cells became markedly rigid losing their deformability, as determined by ektacytometry. Nevertheless, no bud formation was observed in red cell morphology under light microscopy, the MCHC was unchanged with respect to normal cell water content, normal spectrin function was observed, and the mechanical stability of the Triton shells was normal.

The cytoskeletal network appears to be linked normally to the lipid bilayer mostly via band 3 molecules in the presence of the normal amount of protein 4.2 (see Sections 4.1 and 5.1), which has recently been proven to be bound directly to spectrin molecules [81]. However, in the absence of protein 4.2, the cytoskeletal network barely remains nearly normal under an unstressed condition. Once these red

cells are subjected to physicochemical stress, the network easily disassembles by losing its connection with the lipid bilayer. This seems to be a feasible interpretation for the abnormal red cell deformability in the protein 4.2-deficient red cells.

16.2.2.5 Biophysical characteristics

a) Extractability of band 3 The extractability of band 3 has been examined in protein 4.2 deficient red cells. The patients' red cell ghosts were subjected to Triton X-100 (0.3–1.0 %) at pH 8.0 at 4 °C for 30 min. After the incubation, band 3 extracted from the red cell ghosts was examined on SDS–PAGE. The extractability of band 3 from the white ghosts was expressed as the ratio of the amount of band 3 remaining in the white ghosts to the amount of actin. The extractability in the protein 4.2 deficient red cells was $43.3 \pm 4.6 \%$ in 0.3 % Triton X-100, $66.3 \pm 6.0 \%$ in 0.5 % and $76.7 \pm 3.5 \%$ in 1.0 %, compared with normal subjects ($27.0 \pm 2.9 \%$, $42.2 \pm 6.9 \%$ and $60.9 \pm 4.3 \%$, respectively). Therefore, the extractability was enhanced significantly up to 60 % of the normal control.

These observations were also confirmed by other investigators [43]. Skeletons from protein 4.2 deficient red cells retained a greater fraction of band 3 protein than did skeletons from control cells after low-salt (0 mM NaCl) extraction. In contrast, equal fractions of band 3 protein were retained in skeletons from control and protein 4.2 deficient red cells after high-salt (150 mM NaCl) extraction. These results were consistent with the hypothesis that protein 4.2 deficient red cells are depleted specifically from band 3 molecules that are either unattached or bound with low affinity to the membrane skeleton.

Rybicki et al. [46] extracted band 3 using the nonionic detergent octyl- β -glucoside, which selectively extracts band 3 that is not attached to the cytoskeleton and has been used to distinguish free band 3 from cytoskeleton-bound band 3. A 1 % octyl- β -glucoside detergent extracted 30 and 60 % more band 3 from protein 4.2 Nippon and band 3 Montefiore, respectively, which demonstrated the absence or substantial reduction of protein 4.2.

b) Binding of ankyrin to band 3 The inside-out vesicles (IOV) were prepared from protein 4.2 deficient red cells, and then ankyrin depleted IOV was prepared. Ankyrin extracted from normal subjects was added to the ankyrin-depleted IOV, which was prepared from normal and protein 4.2 deficient red cells. The amount of ankyrin that was rebound to the patients' IOV was essentially normal.

Other investigators noted that elutability of ankyrin existed in protein 4.2 deficiency, implying that protein 4.2 may stabilize ankyrin to bind to band 3 [33].

Red cell membranes in homozygous normoblastosis (nb/nb) mice have also been shown to be severely (up to 73 %) protein 4.2 deficient [44]. Reconstitution of nb/nb IOVs with human red cell ankyrin restored ankyrin levels to 80 % of that of normal IOVs, and increased binding of exogenously added human red cell protein 4.2 by 60 %. Therefore, ankyrin may be required for normal associations of protein 4.2 with the red cell membrane.

The role of ankyrin in the formation and stabilization of the spectrin-based skeletal meshwork and of band 3 oligomers was also studied. The results demonstrated that ankyrin was not required for the formation of a stable two-dimensional spectrin-based skeleton although it plays a major role in strengthening the attachment of the skeleton to the membrane bilayer. It is likely that ankyrin is required for the formation of stable band 3 tetramers [45]. The instability of ankyrin in protein 4.2 deficiency, as described above, may be due to a secondary phenomena, in which essentially normal band 3 molecules become unstable in protein 4.2 deficiency, resulting in the instability of ankyrin.

c) Lateral mobility of band 3 Band 3 lateral mobility is constrained in normal human red cell membranes by steric hindrance interactions, low affinity binding interactions, and high-affinity binding interactions [43] (see Section 5.1.2.5). Steric hindrance interactions between band 3 oligomers and the spectrin-based membrane skeleton put major constraints on the laterally mobile band 3 fraction, slowing the rate of band 3 lateral diffusion by approximately 50-fold compared with the predicted diffusion rate of free band 3 in membranes devoid of a functional membrane skeleton. The spectrin/band 3 ratio is the major determinant of the lateral diffusion rate of band 3 [43].

Fluorescence recovery after use of the photobleaching (FRAP) method has shown a shift in the lateral mobility of band 3 consistent with an increase in the mobile fraction of band 3 in membranes from individuals completely lacking protein 4.2 (Fig. 16.10). The total recovery (mobile fraction), as shown, dramatically increased up to almost 100% with almost complete absence of the immobile component of band 3, as compared with normal subjects, in whom the mobile fraction was 0.43 ± 0.11 with a lateral diffusion coefficient of $(6.86 \pm 1.37) \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$. Another common feature of the FRAP curves in these patients is that an almost linear slow recovery component has appeared in addition to the fast recovery component observed in normal red cell ghosts. These abnormal recovery curves suggest that the almost linear slow recovery is not due to simple diffusion of band 3. Since the immobile component in normal ghosts is assigned to the band 3 that is bound to ankyrin, the recovery curves suggest that, in protein 4.2 deficiency, the band 3 that was bound to ankyrin was mobile in the FRAP measurements. This, in turn, suggests that, in the absence of protein 4.2, dissociation of band 3 from ankyrin and reassociation of band 3 to ankyrin occurs frequently, and is detected as a slow mobile fraction in FRAP measurements [30, 31, 35]. In contrast, in the presence of protein 4.2 in normal red cell ghosts, binding of band 3 to ankyrin is stable, dissociation does not occur during FRAP measurements, and the band 3 that is bound to ankyrin is detected as an immobile component. In addition, the fraction of the fast mobile component of band 3 has been reported to be somewhat decreased in protein 4.2 deficient ghosts [30, 31, 35]. This change may be due to increased oligomerization of band 3, which was suggested by increased larger intramembrane particles, since it has been shown that increased oligomerization of band 3 decreases the mobile fraction of FRAP curves of band 3 in normal red cell ghosts.

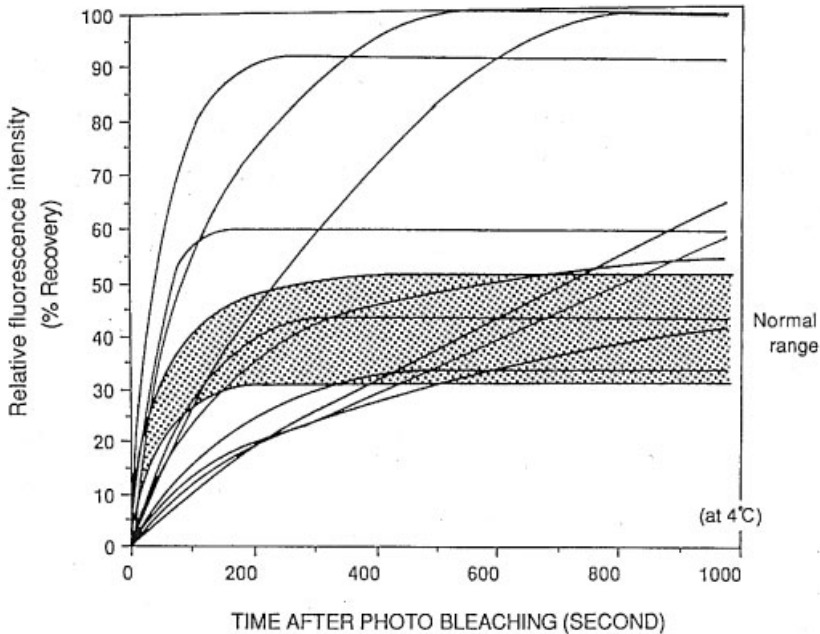


Figure 16.10 Marked increase of a mobile fraction of band 3 with much slower recovery in the red cells of the protein 4.2 deficiency examined by the fluorescence recovery after the photo-bleaching method. A shaded area denotes a normal range.

This observation has been further extended and reconfirmed by other investigators [43]. The lateral diffusion coefficient of band 3 in membranes of protein 4.2 deficient red cells was approximately two-fold greater than the control values. In contrast, the lateral mobility of neither glycophorins nor a fluorescent phospholipid analog was altered in protein 4.2 deficient red cells. The increase in the band 3 lateral diffusion coefficient suggests that low-affinity binding interactions are significantly perturbed in protein 4.2 deficient red cells, and it is likely that the absence of protein 4.2 results directly in a decreased number of low-affinity binding sites for band 3 on the membrane skeleton [43].

Rotational mobility of band 3 Band 3 rotational mobility is constrained in normal red cell membranes by low-affinity and high-affinity binding interactions (see Section 5.1.2.5). The rotationally immobile band 3 fraction apparently represents individual band 3 molecules bound with high affinity to ankyrin. The rapidly rotating band 3 fraction consists of dimers, tetramers, and higher order oligomers of band 3 that are free from rotational constraints other than the viscosity of the lipid bilayer. The slowly rotating band 3 fraction is less well-defined. Rotational constraints applied by low-affinity binding interactions between ankyrin-linked band 3 and other band 3 molecules, and between the cytoplasmic domain of band 3 and membrane skeletal proteins (ankyrin, protein 4.1 and protein 4.2) have been invoked. Steric

hindrance interactions are not important in constraining band 3 rotational mobility.

The rotational mobility of band 3 in protein 4.2 deficient red cells has been studied by three groups [43, 46, 47].

Golan et al. [43] reported that, compared with control red cells, protein 4.2 deficient red cells manifested a significant shift from slowly rotating and rotationally immobile populations of band 3 to a rapidly rotating population, consistent with the interpretation that low-affinity binding sites for band 3 are decreased on the membrane skeleton of protein 4.2 deficient red cells. A significant increase was also noted in the correlation time of the rapidly rotating band 3 population in protein 4.2 deficient red cells compared with control red cells. These results indicate that there is a shift from a lower to a higher order state of band 3 oligomerization in protein 4.2 deficient red cells, and that free band 3 dimers are lost preferentially in these red cells, leading to a larger average size of intramembrane particles visualized by freeze fracture electron microscopy [35, 48]. These results suggest that protein 4.2 deficiency acts primarily to destabilize a fraction of band 3 molecules, resulting in loss of band 3 and possibly of the membrane.

The same observations were made by Rybicki et al. [46]. Band 3 in both protein 4.2 Nippon (>99% protein 4.2 deficient) and band 3 Montefiore (~88% protein 4.2 deficient) ghost membranes showed an increased rotational freedom as compared with band 3 in control ghosts. The primary effect was a shift from slower-rotating and immobile fractions to more mobile, faster decay terms for these two phenotypes of protein 4.2 deficiency.

Contrary to these two reports [43, 46], Wyatt and Cherry [47] concluded that protein 4.2 had no effect on the rotational mobility of band 3. Possible reasons for these discrepancies may be a difference in the experimental conditions. In the cases of Wyatt and Cherry, protein 4.2 was not completely depleted, whereas, in the two other reports, protein 4.2 was almost totally absent.

16.2.2.6 Membrane Transport

a) Sodium transport Sodium transport (see Section 2.3.4.3, Various channels) was examined in red cells washed with 0.154 M Na/K phosphate-buffered saline solution (pH 7.4) and glucose (250 mg dL⁻¹). After incubation of the red cells with ²²Na at 37 °C for 2 h, the radioactivity of the ²²Na remaining in the incubated red cells was counted (sodium influx). Na efflux was determined by incubating the ²²Na-labeled red cells at 37 °C for a further 2 h in the presence or absence of 1.08 mM ouabain in buffer not containing ²²Na. The extent of Na efflux was calculated from the radioactivities in red cells before and after incubation. Red cell sodium and potassium were determined by flame spectrophotometry.

Sodium influx was moderately increased in the protein 4.2 deficient red cells (1.80 ± 0.32 mM L⁻¹ red cells per hour) [30, 31, 35] compared with normal subjects (1.29 ± 0.14). Under the same conditions, Na influx was, respectively, 2.35 ± 0.45 and 1.96 ± 0.34 before and after splenectomy in patients with hereditary spherocytosis (HS).

Total sodium efflux was markedly increased in the protein 4.2 deficient red cells ($7.2 \pm 1.1 \text{ mM L}^{-1}$ red cells per hour) [30, 31, 35] compared with normal subjects (2.4 ± 0.5). The increment was much more striking than that in the conditions before (4.4 ± 1.1) and after (3.6 ± 0.8) splenectomy in classical HS. Na efflux in the protein 4.2 deficiency was increased in both the ouabain-sensitive and ouabain-insensitive sodium efflux.

Red cell sodium [Na] content was significantly elevated ($15 \pm 4 \text{ mM}$) as compared with normal subjects (10 ± 4), the same as it was in unsplenectomized HS patients (16 ± 7) [30, 31, 35]. Red cell potassium [K] content was diminished ($81 \pm 8 \text{ mM}$) as compared with normal subjects (90 ± 5), the same as it was in unsplenectomized HS patients (82 ± 9) with overt hemolysis.

Ouabain-sensitive Na efflux acting as the Na/K pump in the red cells of protein 4.2 deficiency has recently been shown to be increased ($21.4 \pm 1.5 \text{ mmol kg}^{-1} \text{ Hb}^{-1} \cdot \text{h}$), compared with 15.2 ± 1.9 in normal controls [49]. Bumetanide-sensitive Na efflux and bumetanide-sensitive K efflux, and Na/Li exchange, as Na/K/2Cl cotransport, were basically normal [49]. Volume-chloride K efflux as the K/Cl cotransport was $3.6 \pm 0.3 \text{ mmol kg}^{-1} \text{ Hb}^{-1} \cdot \text{h}$ in protein 4.2-deficiency, compared with 6.5 ± 1.2 in normal subjects [49]. Overall Na influx and K efflux as the membrane passive permeability were 33.1 ± 4.1 and $7.2 \pm 2.3 \text{ mmol kg}^{-1} \text{ Hb}^{-1} \cdot \text{h}$ in protein 4.2 deficiency, compared with 15.4 ± 2.7 and 2.1 ± 0.2 in normal subjects [49]. Red cell sodium content ($38.1 \pm 2.3 \text{ mmol kg}^{-1} \text{ Hb}^{-1}$) was increased and potassium content ($230 \pm 6 \text{ mmol kg}^{-1} \text{ Hb}^{-1}$) were decreased in protein 4.2 deficiency, compared with Na (27.2 ± 2.3) and K (287 ± 10) in normal subjects [49]. Increased membrane passive permeability to cations was observed to the same extent amongst HS with band 3 deficiency, with ankyrin and spectrin deficiency, or with protein 4.2 deficiency [49], independent of a specific membrane protein defect. Therefore, it may be speculated that cytoskeletal dysfunction *per se* may alter the permeability barrier of the red cell membrane.

The above-mentioned interpretation is supported by increased cation membrane permeability, which has been reported in various spherocytic mouse red cells [50]. Red cell sodium contents in homozygous $\text{sph}^{\text{ha}}/\text{sph}^{\text{ha}}$, sph/sph , and nb/nb mice were 30.1 ± 0.9 , 28.9 ± 0.3 , and $26.9 \pm 1.5 \text{ mmol L}^{-1}$ red cells, respectively, compared with normal subjects (11.3 ± 0.7) [50]. Red cell potassium contents were 102 ± 2.6 , 101 ± 7.8 , and 97.4 ± 3.0 , compared with normal subjects (123 ± 10) [50]. It has been found that sph/sph exhibit only a trace of spectrin α -chain with defective transcription, processing or stability of α -spectrin mRNA, that $\text{sph}^{\text{ha}}/\text{sph}^{\text{ha}}$ apparently produces an unstable α -chain with 20 to 30 % of the normal spectrin complement and somewhat more β -spectrin than α -spectrin present, and that nb/nb results in reduced levels of ankyrin mRNA with almost complete absence of the 210 kDa protein in red cells and about a 50 % decrease in spectrin, presumably secondary to the loss of spectrin binding sites. Sodium uptake by red cells was 14.8 ± 1.6 , 15.4 ± 3.3 , and $14.7 \pm 3.1 \text{ mmol L}^{-1}$ red cells per hour in $\text{sph}^{\text{ha}}/\text{sph}^{\text{ha}}$, sph/sph , and nb/nb mutants, compared with normal subjects (3.9 ± 1.0). Potassium loss from red cells was 17.0 ± 4.0 , 15.0 ± 3.8 , and 14.1 ± 2.6 in $\text{sph}^{\text{ha}}/\text{sph}^{\text{ha}}$, sph/sph , and nb/nb , compared with normal subjects (6.0 ± 2.1) [50]. The red

cells of these mutant mice with dysfunctional membrane skeletons have increased passive permeability to monovalent cations, suggesting that the membrane skeleton may be critical for maintenance of the membrane permeability barrier.

In the red cells of human protein 4.2 deficiency, a marked derangement of the cytoskeletal network has been verified by electron microscopic studies. The cation transport abnormalities may be produced by an abnormal cytoskeletal network due to the total absence of protein 4.2.

b) Anion transport Red cell anion exchange (see Section 5.1.2.4) has been shown to be sensitive to inhibition by stilbenedisulfonates. Band 3 has two distinct domains, i.e., the cytoplasmic domain and the transmembrane domain. The 55 kDa transmembrane domain contains an anion-transporting site and can mediate anion exchange independently of the cytoplasmic domain. The cytoplasmic domain also has a distinct binding site for protein 4.2. Increasing amounts of protein 4.2 complexed with band 3 have been shown to cause a decrease in band 3 mediated anion transport [51]. The inhibitory effect of protein 4.2 on band 3 mediated anion transport appears to be specific. The specific interaction of protein 4.2 with the cytoplasmic domain of band 3 causes reduction of its anion transport capacity. Protein 4.2 appears to have a possible heterotropic allosteric modulator of band 3 anion transport [51].

In fact, a marked increase in diisothiocyanodehydrostilbene disulfonate (H_2DIDS) sensitive sulfate influx has been observed in the red cells of protein 4.2 deficiency, while no remarkable changes in maximal inhibitory H_2DIDS concentration between normal subjects and protein 4.2 deficient patients is evident [49]. Maximal sulfate influx in protein 4.2 deficiency ranged between 19.8 and 47.4 mmol sulfate per 10^{13} cells·h compared with normal subjects (11.4–14.1). Maximal inhibitory H_2DIDS concentration in protein 4.2 deficiency was from 2.0 to 2.5 μM , compared with normal controls (2.5 to 3.0). Therefore, the ratio between sulfate flux and the maximal inhibitory H_2DIDS concentration ranged from 8.6 to 20.6, in protein 4.2 deficiency compared with normal subjects (4.3 to 4.7), indicating a four-fold increase in activity of the anion transport by band 3 molecules [49]. Therefore protein 4.2 may act as a negative modulator of band 3 mediated anion transport.

However, another study has reported that anion transport in protein 4.2 deficient red cells was normal, and that protein 4.2 did not appear to be required for band 3 anion transport activity [46]. In this study, the calculated maximal rate (V_{max}) and K_m from the Michaelis–Menton equation in protein 4.2 Nippon were $0.24 \times 0.02 \mu mol mL^{-1}$ packed red cells per minutes and $7.0 \pm 1.8 mmol L^{-1}$, compared with 0.25 ± 0.02 and 6.2 ± 1.3 in normal subjects [46]. Therefore, the role of protein 4.2 in anion transport by band 3 still remains to be elucidated.

16.2.2.7 Ultrastructure of Red Cell Membranes *In Situ*

Electron microscopic studies have been carried out to elucidate the abnormalities of red cell membrane ultrastructure *in situ* in total protein 4.2 deficiency [48]. For this purpose, three independent probands were selected, i.e.: 1) a clinically most severely affected protein 4.2 Komatsu [38] proband with a 175 GAT→TAT mutation (in exon 4) with the total absence of protein 4.2 protein, 2) a moderately severely affected protein 4.2 Nippon [35] proband with a 142 Ala→Thr mutation (in exon 3) with an almost missing protein 4.2 protein, and 3) a protein 4.2 Shiga [37], a compound heterozygote with a 317 Arg→Cys mutation (in exon 7) and 142 Ala→Thr (in exon 3) *in trans* with protein 4.2 protein in a trace amount, with rather mild clinical severity.

a) Abnormalities of intramembrane particles (IMPs) Intact red cells were subjected to electron microscopic studies using the freeze fracture method (see Section 3.2.3). In normal subjects, the number of IMPs was $5210 \pm 389 \mu\text{m}^{-2}$, of which approximately 80 % were basically small (4–8 nm) in size. In the red cells of protein 4.2 deficiency of the three types, on the other hand, the number of IMPs had decreased to $4464 \pm 353 \mu\text{m}^{-2}$ in the Nippon type, 4625 ± 381 in protein 4.2 Shiga, and 2975 ± 310 in protein 4.2 Komatsu [48] (Fig. 16.11). Therefore, the decrement in the number of IMPs was most marked in protein 4.2 Komatsu. The decreased number of IMPs appeared to be derived from a decreased number of IMPs of small size in association with an increased number of IMPs of medium (9–20 nm) and large size (>21 nm). The condition of IMPs was found to be most affected in protein 4.2 Komatsu, as judged by a decreased number of

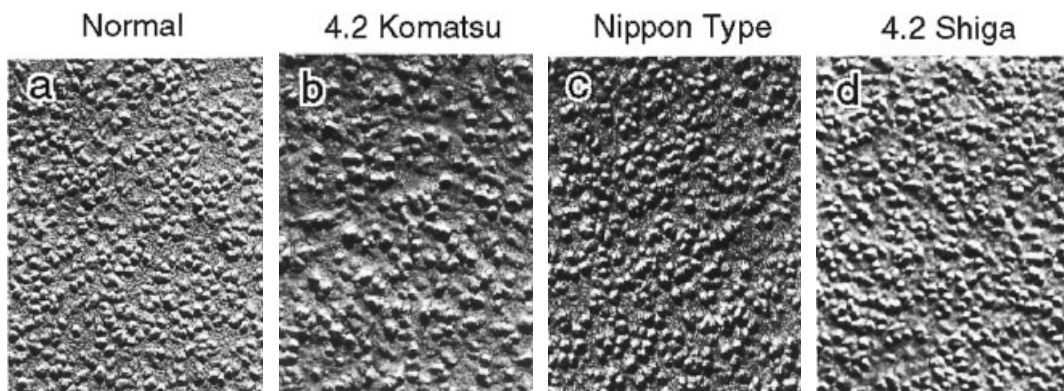


Figure 16.11 Electron micrographs of red cell membranes made by the freeze fracture method showing the distribution and size of IMPs in red cells with protein 4.2 deficiencies. The decreased number and increased size of IMPs are shown representatively in protein 4.2 Komatsu, protein 4.2 Nippon, and protein 4.2

Shiga, compared with the normal controls (far left). Increased sizing of each IMP associated with a decrease in the number is clearly observed to the greatest extent in protein 4.2 Komatsu, and to a lesser extent in protein 4.2 Nippon and least in protein 4.2 Shiga.

IMPs with a marked shift to ones of a large size, probably indicating increased oligomerization of band 3 *in situ* [48]. The extent of the abnormalities was less in protein 4.2 Nippon and the least in protein 4.2 Shiga, although these abnormalities were still demonstrated more than they were in normal controls. It could be speculated that the total absence of protein 4.2 in protein 4.2 Komatsu may produce the most serious derangement in its interaction with band 3 molecules [48]. There may be less serious effects with the other two mutations because of the presence of protein 4.2 even in a trace amount. Another speculation can be made that codon 175 of protein 4.2, as seen in protein 4.2 Komatsu, may be most important as the binding site of protein 4.2 to band 3, and that other codons (142 or 317) as in protein 4.2 Nippon or protein 4.2 Shiga may be important, but to a lesser extent.

The significant contribution of protein 4.2 to the biophysical properties of band 3 was proven by utilizing inside-out vesicles (IOVs) of normal controls and those of protein 4.2 deficiency [48]. In protein 4.2 deficiency, the distribution pattern of IMPs was totally deranged in IOVs, which were prepared from red cell ghosts of protein 4.2 Nippon, compared with those in the normal controls. When spectrins and membrane proteins other than band 3 were stripped from the IOVs at pH 11 in the normal controls, this experimentally produced 4.2 deficiency demonstrated a markedly abnormal aggregation of band 3, which was the same as that in protein 4.2 deficient patients [48]. Therefore, it is clear that protein 4.2 should have the ability to maintain normal distribution of IMPs *in situ*, in which band 3 accounts for approximately 80%.

b) Abnormalities of the cytoskeletal network The cytoskeletal network has also been examined by electron microscopy using the quick-freeze deep-etching method (QFDE) method [48] (see Section 3.2.2). This procedure demonstrated that the filaments (mostly spectrins) of the intact cytoskeletal network in normal subjects were present in multistereotactic dimensions rather than in a single plane. The filaments in the normal subjects were 48 ± 9 nm in length and 7 ± 1 nm in diameter, and appeared to be in a folded configuration [48]. The cytoskeletal network in normal red cells showed a fairly uniform distribution of filamentous structures and also uniformity of apparent branchpoints of the filamentous elements in an essentially orderly fashion. The cytoskeletal network in the normal subjects showed numerous basic units, resembling “cages”, the number of which was $539 \pm 20 \mu\text{m}^{-2}$. The “cage”-like structures consisted essentially of two major types of units, i.e., small (20–44 nm) and medium (45–68 nm) sized units as determined by the interdistance (or diameter) of the longer axis of each structure. In the normal subjects, two-thirds of these units were of small size ($66 \pm 9\%$), and the remaining one-third were of medium size ($30 \pm 6\%$). There were only a few large sized units ($4 \pm 1\%$) in the normal subjects [48].

In contrast, in protein 4.2 deficiency, the uniform distribution of filamentous structures was lost, and apparent branchpoints of the filamentous elements were markedly distorted or disrupted. The extent of the abnormalities of the cytoskeletal network appeared most marked in protein 4.2 Komatsu, and less in protein 4.2

Nippon and protein 4.2 Shiga [48] (Fig. 16.12). The abnormalities of the cytoskeletal network in protein 4.2 deficiencies were semiquantitated by counting the number of apparent cytoskeletal units still left as almost recognizable and tolerable for these counting procedures. The number of these cytoskeletal units was markedly reduced in 4.2 Komatsu ($195 \pm 38 \mu\text{m}^{-2}$), less in Nippon type ($282 \pm 27 \mu\text{m}^{-2}$), and least in 4.2 Shiga ($339 \pm 35 \mu\text{m}^{-2}$) [48].

The relative size distribution of these cytoskeletal units was also semiquantitated by measuring the interdistance (or diameter) of the longer axis in each unit. In protein 4.2 deficiencies, the cytoskeletal units of basic small size (20–44 nm) were markedly reduced in 4.2 Komatsu ($25 \pm 4\%$), in Nippon type ($27 \pm 5\%$), and in 4.2 Shiga ($34 \pm 5\%$), compared with the normal controls ($66 \pm 9\%$) [48]. In their place, units of large size (69–92 nm) and of extra-large size (93–180 nm), which were essentially not present in the normal subjects, were greatly increased in these protein 4.2 deficiencies [48].

It is worth noting that the cytoskeletal network was markedly disrupted in the protein 4.2 deficiencies, as evaluated by the number of cytoskeletal units left, the relative size distribution of these units, and the discontinuation of fibrous filaments. The most striking abnormality of the cytoskeletal network was the disruption of the basic units of the network, as evaluated by the decreased number

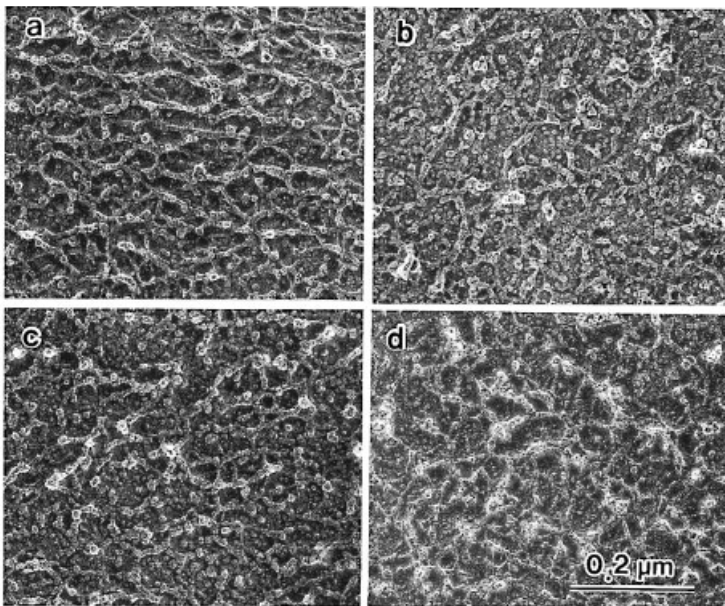


Figure 16.12 Electron micrographs of red cell membrane skeletons made by the quick-freeze deep-etching method in red cells with protein 4.2 deficiencies. Markedly disrupted cytoskeletal networks are shown representatively in protein 4.2 Komatsu (b), protein 4.2 Nippon (c),

and protein 4.2 Shiga (d), compared with the normal control (a). The basic units of the cytoskeletal network were significantly disrupted in protein 4.2 Komatsu, and also distorted in protein 4.2 Nippon and in protein 4.2 Shiga.

of these units in the protein 4.2 deficiencies [48]. The number of the apparent units was decreased to one-third, especially in 4.2 Komatsu, as compared with that in the normal controls [48]. The contents of the cytoskeleton-related membrane proteins (spectrins, ankyrin, actin, and protein 4.1) were essentially normal on SDS–PAGE in these patients with protein 4.2 deficiencies. Therefore the decreased number of apparent basic units of the cytoskeletal network should indicate a marked instability of the network under conditions of the absence of protein. This interpretation is supported by findings regarding the relative size distribution of the cytoskeletal units, as judged by the interdistance (or diameter) of the longer axis of these units. In the protein 4.2 deficiencies, the units of small size (20–44 nm), which are the major structure (as 66 % of the total units in number) under normal conditions, were reduced to only 25–34 % of the total units, in association with a marked increment of large (69–92 nm) units and even of extra-large (93–180 nm) sized units. The disruption was the most marked in protein 4.2 Komatsu, and less in protein 4.2 Nippon and protein 4.2 Shiga [48].

c) Important role of protein 4.2 in stabilizing the cytoskeletal network by its binding to band 3

Under normal conditions, the cytoskeletal network is believed to be stabilized by binding to band 3 molecules tightly via ankyrin (see Chapters 4, 5 and 6). Two-thirds of band 3 is immobilized by this binding, but the other one-third is mobile and unfixed without binding to the cytoskeletal network. In the absence of protein 4.2, the cytoskeletal network appeared to become extremely unstable due to the loss of the integrity of its basic small units, resulting in disruption of the interconnected structure of the cytoskeletal network [48]. Under this pathological condition with a markedly impaired cytoskeletal network, band 3, two-thirds of which is normally connected with the cytoskeletal network mainly via ankyrin, should lose its binding to the network and become unfixed and mobile. It is known that free band 3 molecules tend to aggregate or cluster. The increased large sizes of the IMPs in the protein 4.2 deficiencies may be the result of aggregation and/or clustering of these increased mobile band 3 molecules, which were initially immobile band 3 bound to the cytoskeletal network [48]. The aggregated or clustered band 3 should naturally produce a decrease in the apparent number of IMPs.

It has also been shown that band 3 has binding properties to ankyrin, which binds to β -spectrin (see Section 5.1.2). The bindings probably enhance the vertical stability of the cytoskeletal network. In the three probands with protein 4.2 deficiencies, the contents of the cytoskeletal proteins and of ankyrin were essentially normal. Therefore, the stability of the cytoskeletal network should have been maintained normally in the presence of band 3 and ankyrin, independent of the presence or absence of protein 4.2, if protein 4.2 itself does not have its direct binding to the cytoskeletal network. However, in protein 4.2 deficiency, in which band 3 and ankyrin are present normally, marked instability of the cytoskeletal network was observed [48]. Therefore, it is very likely that protein 4.2 should have its direct binding to the cytoskeletal proteins, especially to spectrins, although the tertiary structure of the protein 4.2 molecule has not been elucidated. A protein 4.2 deficient

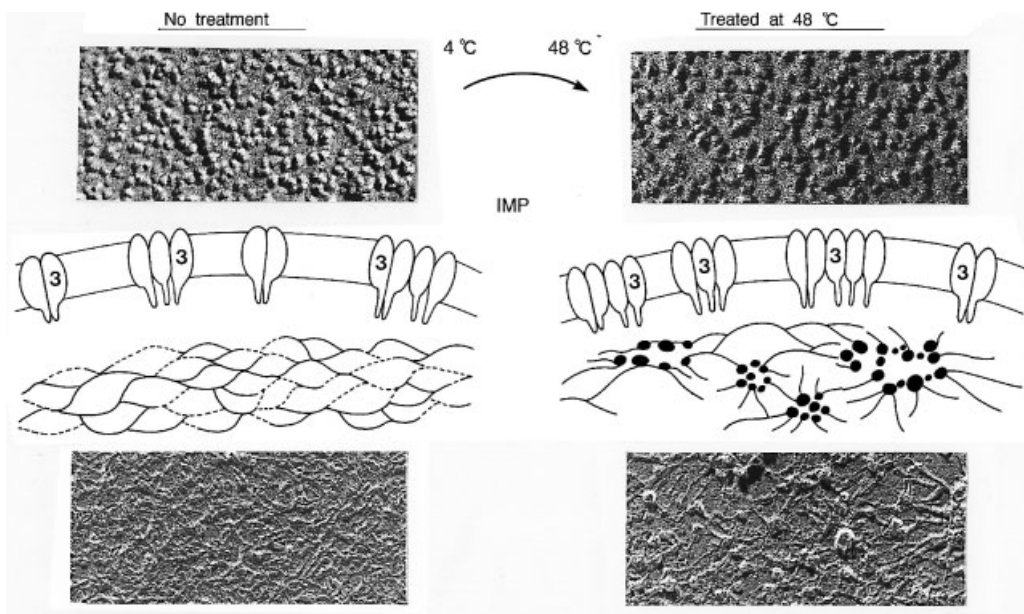


Figure 16.13 Schematic demonstrations of disorganization of the cytoskeletal network and integral protein (band 3) in red cells of protein 4.2 deficiency with the protein 4.2 gene mutation as studied by electron microscopy with the surface replica method and the freeze fracture method. Human intact red cells of homozygous patients with protein 4.2 deficiency were treated by heat at 48 °C for 10 min. The results were compared with those in the same red cells with no heat treatment (4 °C). Intramembrane particles (IMPs) were examined by electron microscopy with the freeze fracture method, and the cytoskeletal network was studied by electron

microscopy with the surface replica method. Ankyrin, protein 4.1 and other membrane proteins except for band 3 are intentionally omitted for better illustrations on IMPs and the cytoskeletal network. Solid lines in this schematic diagram indicate the normal undisrupted cytoskeletal network, and dotted lines indicate the possibly disrupted cytoskeletal network. Dark blobs represent the aggregates of cytoskeletal proteins. Marked aggregates like silver thistles appeared with extensive disruption of cytoskeletal network, as shown in the actual electron micrograph. An increased oligomerization of band 3 is also shown schematically.

state should also produce aggregation or clustering of IMPs, probably by increasing free mobile band 3.

In summary, direct evidence of an impaired cytoskeletal network and of abnormal IMPs was shown in protein 4.2 deficiency by utilizing three probands with different mutations of the protein 4.2 gene. In the structure of protein 4.2, the aspartic acid at codon 175 may be one of the most important factors for maintaining its cellular function, along with alanine at codon 142 and arginine at codon 317. These results clearly indicate that protein 4.2 plays an important role in maintaining the integrity of normal IMPs and a normal cytoskeletal network *in situ* (Fig. 16.13).

16.2.2.8 Protein 4.2 Gene Mutations

a) Protein 4.2 gene mutations in human beings (see Sections 6.2.4 and 10.4) Eight types of total protein 4.2 deficiency due to mutations of the protein 4.2 gene have been identified (Table 16.3) [34, 36–42], i.e., four missense mutations, two frame-shift mutations, one nonsense mutation, and one donor site mutation due to intro-nic substitutions. Therefore, missense mutations are predominant, especially allele protein 4.2 Nippon (142 GCT→ACT: Ala→Thr), which has been observed in 17 patients of 13 kindreds amongst 28 patients of 19 kindreds with complete protein 4.2 deficiency. These protein 4.2 gene mutations have been found mostly or nearly exclusively in the Japanese population, i.e.: protein 4.2 Nippon [33–35], protein 4.2 Shiga [37], protein 4.2 Komatsu [38], protein 4.2 Fukuoka [36], and protein 4.2 Notame [41]. Only three mutations have been observed in the non-Japanese population, i.e.: protein 4.2 Tozeur in Tunisia (310 CGA→CAA) [40] and protein 4.2 Lisboa in Portugal (88 AAG GTG→AAG TG) [39] in addition to a few Italian Caucasian patients who were a homozygous protein 4.2 Nippon and protein 4.2 Nancy in France [42].

Among these protein 4.2 gene mutations, the mutation of the Nippon type (142 GCT→ACT) is most important in the Japanese population, because it is involved in homozygotes of protein 4.2 Nippon, and also in compound heterozygotes of protein 4.2 Shiga with 317 (CGC→TGC), protein 4.2 Fukuoka with 119 (TGG→TGA), and protein 4.2 Notame with G→A at the intron 6 donor site.

The mutations of the protein 4.2 gene appear to be clustered around exon 3 and at the 5' side of exon 7. Interestingly, no mutation has been reported from exon 8 to exon 13 at the C-terminus. Therefore, the regions around exons 3 and 7 could be biologically important as so-called “hot spots”. An experiment with the targeted protein 4.2 gene for its knock-out mice has recently been designed based on these observations [52].

Allele frequency of the Nippon type (142 GCT→ACT) appears to be around 3% in the normal Japanese population [29–31].

Complete protein 4.2 deficiency appears to be transmitted by autosomal recessive inheritance, and most patients have been homozygotes or compound heterozygotes of missense mutations on the protein 4.2 gene [29]. Therefore, sole heterozygotes of these missense mutations have been asymptomatic with nearly normal protein 4.2 content in red cells.

In two patients with protein 4.2 doublet Nagano, in which two protein 4.2 peptides of 72 kDa and 74 kDa were expressed in nearly equal amounts, the mutation of the protein 4.2 gene (488 CGT→CAT in exon 10) was linked to this abnormality by heterozygotes in the family members [53].

b) A null mutation (4.2^{-/-}) of protein 4.2 in mice The red cell membrane protein 4.2 gene (Epb 4.2) has recently been targeted in embryonic stem (ES) cells to create a null mutation (4.2^{-/-}) in mice [52]. The mouse Epb 4.2 is ~22 kb in length and consists of 13 exons (see Section 6.2.4.3). A fragment extending from intron 3 to exon 8 was replaced by a neomycin-resistant cassette, removing exons 4 through

7 and part of exon 8. Homozygous null mutations were not distinguishable from normal littermates by phenotype at any age. Genotyping revealed the expected Mendelian frequency of homozygous null ($4.2^{-/-}$; 23%), heterozygous ($4.2^{+/-}$; 51%), and wild-type ($4.2^{+/+}$; 26%) offspring from heterozygous mating pairs [52].

Protein 4.2 was not detected in $4.2^{-/-}$ -red cell ghosts on Coomassie blue-stained SDS-PAGE gels or by Western blotting [52]. No protein 4.2 mRNA was detected by Northern blot analysis of $4.2^{-/-}$ newborn reticulocyte RNA [52]. The protein 4.2/spectrin ratios were 0.14 ± 0.01 in $4.2^{+/+}$ and 0.10 ± 0.01 in $4.2^{+/-}$, indicating that the content of protein 4.2 in red cells of heterozygotes ($4.2^{+/-}$) was decreased.

Hematologically, $4.2^{-/-}$ mice had mild hereditary spherocytosis (HS) [52]. Red cell counts and hematocrits were significantly reduced in $4.2^{-/-}$ mice ($9.7 \pm 0.2 \times 10^{12} \text{ L}^{-1}$ and $44.7 \pm 0.9\%$ versus $10.4 \pm 0.2 \times 10^{12} \text{ L}^{-1}$ and $51.8 \pm 0.7\%$ in $4.2^{+/+}$ mice). Reticulocyte percentages in $4.2^{-/-}$ mice were $5.5 \pm 0.8\%$ compared with 2.5 ± 0.1 in $4.2^{+/+}$ mice. The mean cell volume (MCV) and mean cell hemoglobin concentration (MCHC) were $46.3 \pm 0.7 \text{ fL}$ and $34.5 \pm 0.5\%$, respectively, compared with $49.9 \pm 0.8 \text{ fL}$ and $31.9 \pm 0.4\%$ in $4.2^{+/+}$ mice. All hematological parameters were normal in $4.2^{+/-}$ mice. Red cell morphology demonstrated the presence of spherocytosis in $4.2^{-/-}$ mice [52], and both normal biconcave red cells and intermediate, cup-shaped cells in $4.2^{+/-}$ mice. The deformability index was lower than normal in $4.2^{+/-}$ red cells and was further decreased in $4.2^{-/-}$ mice [52].

In protein chemistry, the band 3 content of $4.2^{-/-}$ red cells appeared to be decreased on SDS-PAGE gels and Western blots [52]. The band 3/spectrin ratio was 1.00 ± 0.03 in $4.2^{-/-}$ mice and 1.19 ± 0.07 in $4.2^{+/+}$ mice. Net DIDS-sensitive sulfate influx was decreased to $\sim 60\%$ of the wild-type in $4.2^{-/-}$ mice. In $4.2^{+/-}$ red cells, the band 3/spectrin ratio was also decreased (1.12 ± 0.04), and the net DIDS-sensitive sulfate influx was $\sim 80\%$ of normal. In $4.2^{-/-}$ red cells, normal amounts of spectrin, ankyrin, protein 4.1, p55, and glycophorin C were observed.

Ultrastructurally, a normal membrane skeleton has been reported to be assembled in $4.2^{-/-}$ red cells, despite the absence of protein 4.2 and partial band 3 deficiency [52]. However, the decreased number of intramembrane particles (IMPs) that remained were clustered in $4.2^{-/-}$ red cells [52]. It was speculated that this was due to destabilization of the membrane because of a lack of horizontal lipid-protein interactions in those areas that were relatively devoid of integral membrane proteins within the bilayer.

In $4.2^{-/-}$ red cells, the Na^+ content was increased and the K^+ content was decreased [52]. The absolute K^+ loss exceeded the Na^+ again, resulting in dehydration. No abnormalities in the Na^+ and K^+ contents were observed in $4.2^{+/-}$ red cells. The maximal rates of the Na/K pump were identical among the three genotypes. There was a small but significant increase in the activity of the K-Cl cotransporter (Cl-dependent efflux), significant increases in the activities of the bumetanide-sensitive Na-K-2Cl cotransporter and calcium-stimulated K^+ efflux (Gardos) channel, a dramatic increase in the activity of the Na/H exchanger, a small increase in passive Na^+ permeability, and normal passive K^+ permeability in $4.2^{-/-}$ red cells [52]. The increased transport activities and passive Na permeability observed in $4.2^{-/-}$ red cells were due to increased sensitivity to cell shrinkage. It has been

shown that increased Na-K-2Cl cotransporter and Na/H exchanger activities correlate with a net increase in phosphorylation. In $4.2^{-/-}$ red cells, cytosolic protein kinase C (PKC) was significantly decreased with decreased PKC- α and PKC- β I isoforms but normal PKC- β II [52]. Cytosolic protein kinase A (PKA) activity was increased in $4.2^{-/-}$ red cells. Basal phosphorylation was increased and PMA-stimulated phosphorylation was reduced in $4.2^{-/-}$ red cell membranes, in which cytosolic casein kinase I (CKI) activity was normal with decreased cytosolic CKII [52]. The functional significance of these findings remains to be elucidated in the future. In addition, the contribution of partial band 3 deficiency to $4.2^{-/-}$ red cell cation transport is also unknown.

In nonerythroid expression of protein 4.2, protein 4.2 was present in normal platelets and absent from $4.2^{-/-}$ platelets, indicating that protein 4.2 in a platelet was a product of the same gene (*Epb 4.2*) as was red cell protein 4.2 [52]. A normal platelets count, however, was observed ($1112 \pm 130 \times 10^3 \mu\text{L}^{-1}$ in $4.2^{-/-}$, 1177 ± 108 in $4.2^{+/+}$) [52]. Histological examination of brain, spinal cord, heart, lung, liver, kidney, intestine, and muscle revealed no overt pathological change in $4.2^{-/-}$ mice at two or nine months of age [52].

In summary, most of the observations in 4.2 knock-out mice are compatible and confirmatory with those observed previously in human patients with total deficiency of red cell protein 4.2 [29–31, 33–44, 46–48]. Some findings, however, differ from those in human cases, especially in heterozygotes [29–31, 35, 48]. Human heterozygotes were totally silent in clinical hematology with normal red cell indices and normal reticulocyte counts [29–31]. In addition, no abnormalities were observed in red cell membrane proteins, even in protein 4.2 content and red cell cation content [29–31]. These minor discrepancies could be due to a species difference in some part and also to the artificial gene manipulation in the protein 4.2 knock-out mice. Considering the evaluation of the tremendous instability of skeletal network especially under heated conditions, which was observed in human protein 4.2 deficiency [29–31, 35, 48], the authors did not perform these experiments in $4.2^{-/-}$ mice [52].

16.2.2.9 Band 3 Gene Mutations

Band 3 is one of a family of anion exchanger (AE) genes (see Sections 5.1 and 15.1). AE1 is expressed in red cells and also in both mouse and human kidney as an alternative isoform that utilizes a downstream start codon and excludes exons 1 to 3 [54]. These exons encode amino acids involved in membrane skeleton binding [55], and recent evidence confirms that this function is lacking in the truncated kidney isoform [56, 57]. AE2 is ubiquitously expressed but is particularly prominent in the gastrointestinal tract and choroids plexus, while AE3 is expressed in brain neurons, the retina, heart, and kidney [58]. The membrane spanning domains of AE1, AE2, and AE3 are highly conserved and function in anion exchange. The cytoplasmic domains show less conservation. AE2 and AE3 have ~300 additional amino acids at their N-termini compared with AE1 [58].

a) Total protein 4.2 deficiency in homozygous band 3 gene mutations in animal models Total deficiency of protein 4.2 has also been reported in a complete lack of band 3 in red cell membranes, i. e.: (1) in Japanese cattle due to a nonsense mutation of the band 3 gene [59], (2) in knock-out mice by targeted disruption of the band 3 gene [60], and (3) in the knock-out mice also by selectively targeted inactivation of the erythroid band 3 gene, in which kidney band 3 was not affected [61] (see Sections 5.1 and 15.1).

A moderate anemia of autosomal incompletely dominant inheritance with marked microspherocytosis [59] has been reported in Japanese cattle. In these cattle, no band 3 was detected due to a nonsense mutation (CGA→TGA; Arg→stop) of the band 3 gene at the position corresponding to codon 646 in human red cell band 3 cDNA [59]. Immunoblotting analysis demonstrated a very low content or nearly complete absence of protein 4.2 in red cell membranes of the proband. Considerable decreases were also observed in other major red cell membrane components such as spectrin, actin, glyceraldehyde 3-phosphate dehydrogenase (band 6), and ankyrin (a reduction by at least 50% of normal subjects). The probands also exhibited a marked distortion and disruption of the membrane skeletal network with tremendous instability [59].

The affected cattle lacked kidney proteins, which are antigenically related to band 3, as in erythroid cells [59]. The proband red cells completely lacked rapid anion exchange as a function of band 3 protein; i.e., the defective $\text{Cl}^-/\text{HCO}_3^-$ exchange in these cells was uncompensated for and limited to a fairly low level [59].

Targeted disruption of the murine erythroid band 3 gene has resulted in spherocytosis and severe hemolytic anemia [61]. The erythroid band 3 gene was selectively inactivated but not the kidney band 3 gene. Red cells of homozygous mice were completely devoid of band 3 protein, whereas normal levels of band 3 protein were detected in the lysates of kidneys obtained from band 3^{-/-} mice [61]. Densitometric analysis of red cell membrane proteins indicated that the mutant ghosts contained 75% of the normal spectrin, significantly reduced ankyrin (40% of normal) and no detectable protein 4.2 [61]. Normal amounts of protein 4.1 and actin were detected in the red cell membranes of homozygous mice [61]. The presence of a reduced but significant amount of ankyrin in band 3^{-/-} ghosts lends further support to the existence of band 3 independent sites for the attachment of ankyrin in the red cell membrane. Similarly, the presence of normal amounts of protein 4.1 in band 3^{-/-} ghosts indicates that the *in vitro* binding of protein 4.1 with band 3 may not occur *in vivo*. The concurrent loss of protein 4.2 in band 3^{-/-} red cells shows that the binding of protein 4.2 to the plasma membrane is exclusively determined by its interaction with band 3 [61]. The red cell phenotype of the mice of this type is consistent with the results obtained from the cattle with the homozygous nonsense mutation of the band 3 gene [59]. The band 3^{-/-} red cells also contained adducin, dematin, p55, and glycophorin C. In contrast, the band 3^{-/-} red cells are completely devoid of glycophorin A (GPA), although the polymerase chain reaction (PCR) confirmed the presence of GPA mRNA (see Sections 5.1, 5.2, and 15.1).

The function of band 3 was also examined in the mice with targeted mutagenesis [60]. The mouse anion exchanger 1 (AE1) gene consists of 20 exons. A 1130 base

pair segment between exons 9 and 11 was replaced with a neoR cassette. This segment encompasses the distal portion of the N-terminal cytoplasmic domain and the first membrane-spanning segment of the C-terminal domain. In the homozygous targeted mice, no AE1 transcript was detected in newborn reticulocyte of 14.5-day fetal liver RNA using a full-length AE1 cDNA [60]. No protein was detected using antibodies raised against either the cytoplasmic or membrane-spanning domains of AE1 in red cell membranes or in whole cell lysates, confirming the absence of the normal AE1 gene product as well as the absence of any truncated AE1 polypeptides derived from the targeted gene [60]. AE1^{-/-} red cell ghosts contained $84.7 \pm 5.5\%$, $86.4 \pm 5.4\%$, and $48.8 \pm 5.0\%$ of wild type, steady state levels of α -spectrin, β -spectrin, and ankyrin, respectively [60]. The near normal spectrin content in AE1-deficient red cell membranes suggests the possibility of alternative membrane binding sites for spectrin or alternative mechanisms of assembling the membrane skeleton [60]. In AE1^{+/-} red cells, normal amounts of α - and β -spectrin ($94.8 \pm 5.4\%$ and $96.8 \pm 4.2\%$, respectively) and ankyrin ($119 \pm 6.9\%$) but decreased levels of AE1 ($82.3 \pm 2.1\%$) were observed [60].

In AE1^{-/-} red cells, no protein 4.2 was detected, although these red cells retained 50 % of the normal amount of ankyrin [60]. Therefore, it appears that AE1 contains the sole, high affinity binding site for protein 4.2.

b) Total protein 4.2 deficiency in human band 3 gene mutations Three kindred have been reported among patients with band 3 gene mutations in which protein 4.2 was totally or nearly completely missing in their red cells (see Sections 11.3.3, 15.1.3 and 15.1.5).

The first example is a Japanese family with four mutations on the band 3 gene [62]; i.e., two mutations of Memphis II polymorphism (K56E, AAG→GAG, and P854L, CCG→CTG) and, additionally, a mutation (G714R, GGG→AGG) in one allele (allele Okinawa), and, *in trans* to allele Okinawa, a mutation (G130R, GGA→AGA) in another allele (allele Fukuoka). The allele Fukuoka has been known to alter the binding of protein 4.2 to band 3 [63]. The proband (the daughter) presented with a pronounced decrease of band 3 ($49.8 \pm 0.3\%$ of normal), and showed an almost complete lack of protein 4.2 with only traces (less than 0.1 % of normal) of 72, 68 and 66 kDa fragments of protein 4.2. Her mother showed a partial deficiency in band 3 (–25 % on average) and a proportional reduction in protein 4.2. Therefore, the mother was heterozygous for a novel allele of the EPB3 gene, allele Okinawa, and her daughter was a compound heterozygote of allele Okinawa and allele Fukuoka. Heterozygosity for allele Fukuoka has been documented in another individual who showed no clinical or hematological signs, and normal band 3 content [63]. It has been suggested that band 3 Okinawa binds virtually all the protein 4.2 in red cell precursors, band 3 Fukuoka is unable to do so, and that band 3 Okinawa cannot be incorporated into the membrane leading to degradation of the band 3 Okinawa protein complex. In contrast, band 3 Fukuoka, free of bound protein 4.2, could then be incorporated normally into the lipid bilayer [63]. Thus, it has been speculated that protein 4.2 would not appear in the proband's red cell membranes.

The second example of total protein 4.2 deficiency due to human band 3 gene mutation was found in a Portuguese baby with a missense mutation (band 3 Coimbra: V488M) in the homozygous state [64, 65]. In a large Portuguese family, there was a couple whose members carried the mutation Coimbra in the heterozygous state. At the second pregnancy of this couple, homozygosity for mutation Coimbra was ascertained antenatally and the pregnancy was interrupted. At the third pregnancy, a severely anemic hydropic female baby in the homozygous state was reanimated and kept alive with an intensive transfusional regime. Cord blood smears disclosed dramatic erythroblastosis and poikilocytosis. Red cells with a tail-like elongation were a conspicuous feature. Band 3 and protein 4.2 were completely absent in red cell membranes. Metabolic acidosis and nephrocalcinosis were present. The total absence of band 3 in humans appears to be reasonably compatible with life as long as intensive transfusion support is provided. In the heterozygous state, the band 3 content, 4,4'-diisothiocyano-1,2-diphenylethane-2,2'-disulfonate (H_2DIDS) sites ($\mu\text{mol L}^{-1}$), and sulfate flux ($\text{nmol per } 10^8 \text{ red cells per } 10 \text{ min}$) were -23% of normal on average, -35% of normal, and -34% of normal, respectively. Therefore, total protein 4.2 deficiency did occur in the homozygous state of the missense mutation of the band 3 gene.

A third human case of the homozygous hereditary spherocytosis (band 3 Neapolis) due to splicing mutation at position +2 in the donor splice site of intron 2 of the AE1 gene has been reported [66].

16.2.3

Partial Deficiency of Protein 4.2

Partial deficiency of protein 4.2 is fairly common in hereditary spherocytosis with band 3 mutations under the two following situations (see Section 15.1.6).

16.2.3.1 Partial or Total Lack of One Haploid Set of Mutated Band 3

The red cells lack one haploid set (a heterozygous state), partially or totally, of mutant band 3 (20~40% reduction of overall band 3), yielding mild to moderate hereditary spherocytosis with a dominant inheritance pattern. To date, numerous heterogenous mutations have been reported (see Sections 10.4, 11.5 and 15.1.2). As a consequence, protein 4.2 is diminished in roughly the same proportions as band 3.

The first report to examine whether or not the product of the mutant allele is inserted into the membrane utilized one HS subject who was doubly heterozygous for the R760Q mutation and K56E (band 3^{Memphis}) polymorphism of the human band 3 gene [67]. Only band 3^{Memphis} was detected in the red cell membrane, indicating that the protein product of the mutant (R760Q) band 3 allele was absent from the red cell membrane.

The same line of reports appeared in 166 families with autosomal dominant HS. In these families, band 3 deficiency was invariably associated with mild autosomal dominant HS [68]. They detected the first subset of band 3 gene mutations with seven nonsense and frameshift mutations that were all associated with the absence

of the mutant mRNA allele from reticulocyte RNA, implicating decreased production and/or stability of mutant mRNA as the cause of decreased band 3 synthesis [68]. The second subset included five substitutions of highly conserved amino acids and one in-frame deletion, which were associated with the presence of comparable levels of normal and mutant band 3 mRNA [68].

A French 18-year old male demonstrated moderate HS with a 35 % decrease in red cell band 3 content [69]. The underlying mutation was allele Lyon (R150X: CGA→TGA) with allele Genas, which was a G→A substitution at position 62 before codon 1 (G→A). It has been shown: (1) that the allele Genas (father) resulted in a 33 % decrease in the amount of band 3 mRNA, (2) that the reduction caused by the allele Lyon (mother) was 42 %, and (3) that the compound heterozygous state for both alleles (proband) resulted in a 58 % decrease [69].

It has also been shown that a mutant transcript is present in HS patients bearing missense mutations, whereas only the normal transcript is found in HS patients with frameshift mutations, in which the mean decrease in membrane band 3 content is significantly lower, leading to speculation that missense mutations may have some sort of dominant negative effect [70].

Band 3 Foggia (del C; ACCCAC→ACCAC in codon 55) and band 3 Napoli I (298–299 ins T; TCT→TTCT in codon 100) resulted in premature termination of translation, making one haploid set of band 3 mRNA unavailable [71], as on band 3 Milano (Gln plus duplication of residues 478–499) which is probably not incorporated into the membrane [72].

A nonsense mutation (Q330X) of the human red cell band 3 gene has been detected in HS [72]. This mutation was present in genomic DNA. In addition, a marked quantitative decrease in accumulation of the mutant band 3 RNA has been detected [72].

In these situations, the extent of the decrease of protein 4.2 content was basically proportional to that of the band 3 content [73].

16.2.3.2 Mutations in the Cytoplasmic Domain of Band 3, Which Contains Major Binding Sites for Protein 4.2.

The decrement of the protein 4.2 content is disproportionately greater when mutations of the band 3 gene are present in its cytoplasmic domain, where the binding site(s) for protein 4.2 is located; e. g., band 3 Tuscaloosa (P327R: CCC→CGC) [74], band 3 Montefiore (E40K: GAG→AAG) [75], and band 3 Fukuoka (G130R: GGA→AGA) [76]. The inheritance pattern is recessive for band 3 Montefiore and band 3 Fukuoka. In these cases, protein 4.2 is sharply decreased due to the mutations on the band 3 gene.

A partial ($29 \pm 5\%$) deficiency of protein 4.2 was discovered in red cells of hereditary spherocytosis [74], in which one band 3 allele was normal but the other allele contained two mutations of the band 3 gene: (1) band 3 Memphis (K56E: AAG→GAG) and (2) band 3 Tuscaloosa (P327R: CCC→CGC). The predicted maximal binding capacity of the patient's inside-out vesicles (IOVs) for protein 4.2 was 33 % lower than that of control IOVs ($208 \pm 9 \mu\text{g mg}^{-1}$ compared

with $312 \pm 1 \mu\text{g mg}^{-1}$ for control membranes) [74]. The K_d for binding to patient membranes was also decreased nearly two-fold ($2.4 \pm 0.2 \times 10^{-7} \text{ mol L}^{-1}$ compared with $4.6 \pm 0.3 \times 10^{-7} \text{ mol L}^{-1}$ for the control) [74].

A homozygous state for band 3 Montefiore (E40K: GAG→AAG) with a marked (88%) deficiency of protein 4.2 has been reported [75]. In the proband, the *in vitro* binding of protein 4.2 purified from control red cells to the proband's protein 4.2 stripped IOVs was decreased by 30% and 8% in two experiments, compared with normal controls [75]. However, the authors themselves had some reservation on this matter, because these small differences in binding were inconclusive and certainly could not explain the 88% protein 4.2 deficiency in the proband's red cell membranes [75]. Levels of all other membrane proteins in this proband were normal except for band 6 (glyceraldehyde-3-phosphate dehydrogenase), which was 30% decreased [75].

An extremely rare homozygous missense mutation of the band 3 gene (band 3 Fukuoka; G130R: GGA→AGA) showed substantial reduction (45.0% of that of normal subjects) in addition to a minimal reduction (9.3%) of band 3 content [76]. Therefore, the extent of the decrement of protein 4.2 was disproportionately greater than that of band 3 [76]. It is also noteworthy that, in addition to the 72 kDa peptide (a wild type of protein 4.2), a trace amount of the 68 kDa peptide was detected in the proband [76]. The extent of the rebinding of the proband's IOVs to the normal protein 4.2 was markedly reduced, compared with that of normal subjects. Scatchard plots indicated the average rebinding capacity in the proband was 207 μg of protein 4.2 per mg of vesicle proteins versus 295 μg in a normal subject. Therefore, the rebinding capacity of the mutated band 3 Fukuoka to normal protein 4.2 appeared to be reduced to approximately 70% of the normal band 3 [76]. Therefore, the disproportional reduction of protein 4.2 compared with that of band 3 was most likely due to the functional abnormality of the mutated band 3 [76].

16.2.4

Protein 4.2 Doublets

Two independent families with a doublet protein 4.2 in red cells, in which two protein 4.2 isoforms were present, 72 kDa as a wild type and 74 kDa, have been described [53, 77]. The total amounts of protein 4.2, the sum of 72 and 74 kDa, were essentially normal.

The proband of the first family suffered from uncompensated hemolytic anemia with stomatocytosis (Fig. 16.14) [77]. This patient demonstrated a single band at 72 kDa for protein 4.2 in the normal amount. In six out of ten family members, however, protein 4.2 consisted of two forms: 72 and 74 kDa in equal amounts (Fig. 16.15) [77]. These doublet cases (protein 4.2 doublet Kobe) also demonstrated stomatocytosis without any clinical symptoms. Four other family members showed only a single protein 4.2 (72 kDa alone) with stomatocytosis, also without clinical symptoms [77]. In the red cells with the protein 4.2 doublet, sodium influx ($1.5\sim 1.9 \text{ mmol L}^{-1}$ red cells per hour; normal: 1.29 ± 0.14) and sodium efflux ($3\sim 7 \text{ mmol L}^{-1}$ red cells per hour; normal: 2.4 ± 0.5) were slightly enhanced [77].

Figure 16.14 Scanning electron micrograph of peripheral red cells of the patient with protein 4.2 doublet.

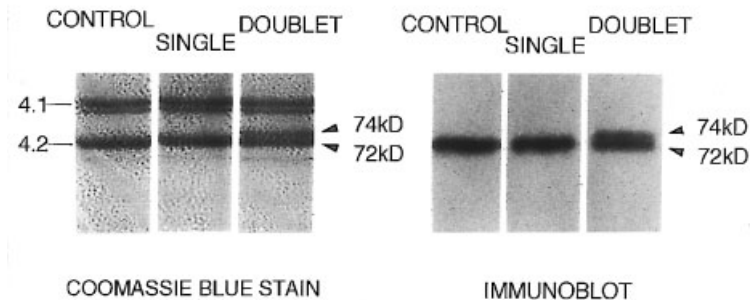
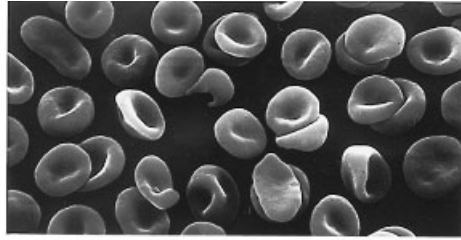


Figure 16.15 A doublet protein 4.2 (72 kDa + 74 kDa) disease as a variant of protein 4.2 anomalies. In the addition of the wild type (72 kDa) of protein 4.2 peptide (in CONTROL), a 74 kDa variant of protein 4.2 is also present in the individuals of a doublet protein 4.2 disease (DOUBLET). In this family, some members indicate only a single band (SINGLE) with protein 4.2 of a wild type (72 kDa). The representative results by Coomassie blue staining (left) and by immunoblot (right) with anti-human protein 4.2 polyclonal antibody are shown.

Ektacytometry revealed normal rheological properties in the fresh intact red cells of these patients [77].

The second family (two cases) also demonstrated a doublet with 72 and 74 kDa, but the distribution of these two isoforms was 70 % for 72 kDa and 30 % for 74 kDa (protein 4.2 doublet Nagano) [53]. The sum of the 72 and 74 kDa peptides was equivalent to the normal control, in which only the 72 kDa was present as a wild type. These patients showed overt hemolysis with marked reticulocytosis and increased MCHC [53]. The patient's red cell morphology demonstrated marked stomatocytosis and the presence of target cells. The two patients also demonstrated a marked increase in red cell membrane phosphatidylcholine, which would be responsible for target cells in the peripheral blood [53]. Full sequencing of the protein 4.2 gene by RT-PCR yielded only a normal gene size corresponding to its wild type (72 kDa) with a heterozygous mutation of R488H [53], which was confirmed in genomic DNA. The mutation was linked to the protein 4.2 doublet anomaly in the proband and his daughter, and not found in other family members, including a brother with normal 72 kDa and membrane lipid anomalies [53]. The 74 kDa peptide of protein 4.2 appears to be derived from the protein 4.2 (72 kDa) gene by the posttranslational modification, because the 90 nucleotide segment in exon 1 of the protein 4.2 gene was skipped, as is usually observed in the wild

type of protein 4.2 (72 kDa) contrary to expectations, implying that the 74 kDa was not produced by devoting of the normal skipping of the 90 nucleotides in exon 1. The intramembrane particles and skeletal network were virtually unaffected, as examined by electron microscopy [53]. Concomitantly, red cell membrane lipid analysis in the proband revealed markedly increased free cholesterol (FC) ($1681 \mu\text{g per } 10^{10}$ red cells; normal: 1202 ± 103), and phosphatidylcholine (PC) ($1063 \mu\text{g per } 10^{10}$ red cells; normal: 733 ± 64) [53]. The content of FC was $1384 \mu\text{g per } 10^{10}$ RBC in the brother and $1413 \mu\text{g per } 10^{10}$ red cells in the daughter, and that of PC was $943 \mu\text{g per } 10^{10}$ red cells in the brother and 865 in the daughter. The composition of other phospholipids was essentially normal. Other family members demonstrated no abnormalities of membrane proteins or membrane lipids [53]. Therefore, the membrane lipid abnormalities were proven not to be linked to the protein 4.2 doublet in the proband and his daughter, because his brother demonstrated the membrane lipid abnormalities in the absence of the protein 4.2 doublet [53].

Protein 4.2 isoforms have reportedly been observed in various animals [78–80]. In Oriental deer (*Cervus taiouanus* and *Cervus Nippon yesoensis*, Heud), 80 % of a wild type consisted of a 78 kDa peptide. As an isoform of protein 4.2, a 76 kDa peptide was also present. In five out of 25 deer, a protein 4.2 doublet was detected, in which 78 kDa peptides were present in equal amounts.

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17

Abnormalities of Membrane Lipids

17.1

Introduction

Membrane lipids compose approximately 50% by weight of the mature red cell membranes [1, 2] (see Section 2.2.1). Free unesterified cholesterol and phospholipids are predominant, and are present in nearly equal proportions; that is, the molar ratio of cholesterol:phospholipids is 0.80. Small amounts of glycolipids are also present. Regarding phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM), and phosphatidylserine (PS) are the predominant phospholipids. Small amounts of phosphatidic acid (PA), phosphatidylinositol (PI), and lysophosphatidylcholine (lyso-PC) are also present in the red cell membranes. At the physiological pH, phosphatidylserine, phosphatidic acid, and phosphatidylinositol exhibit a net negative charge, whereas the other phospholipids are electrically neutral. Most of lipids except sphingomyelin and lysophosphatidylcholine have two fatty acids which are attached to a glycerol main structure. The lysophosphatidylcholine has only one fatty acid chain, and exhibits the hemolytic effect because of its detergent-like nature.

The phospholipids in the red cell membrane are in a planar bilayer with their polar head groups exposed at each surface and their hydrophobic fatty acyl side chains buried in the bilayer core (see Section 3.1). Glycolipids and cholesterol are intercalated between the phospholipids in the bilayer, with their long axes perpendicular to the bilayer plane. Red cell glycolipids are located entirely in the external half of the bilayer with their carbohydrate moieties extending into the aqueous phase. These glycolipids carry several important red cell antigens (see Section 5.3).

The red cell membrane lipids are asymmetrically distributed across the bilayer plane, which is known as *trans* asymmetry (see Section 2.2.2). This asymmetric distribution of phospholipids reflects a steady state involving a constant exchange of phospholipids by a flip/flop mechanism between the two bilayer membrane lipid leaflets. The transmembrane shuttle of these phospholipids in biological membranes is extremely fast (see Section 2.2.4). The phospholipid flip and flop is accelerated by transmembrane proteins, which produce localized discontinuities in the bilayer, or by transmembrane pH gradients in the case of neutral phospholipids (see Section 2.2.2). The *trans* asymmetry of phospholipids is produced and maintained by an adenosine triphosphate (ATP) dependent transport system, i. e., the amino

phospholipid translocase (so-called flippase), which translocates phosphatidylserine and phosphatidylethanolamine from the outer leaflet to the inner leaflet. The flippase is a 130 kDa integral membrane protein which is a member of the Mg^{2+} -dependent P-glycoprotein ATPases. For the flop mechanism, the presence of floppase is suspected, because phosphatidylcholine and sphingomyelin are not transported inward by the flippase enzyme, and red cells are able to transport phosphatidylcholine, along with phosphatidylserine and phosphatidylethanolamine from the inner leaflet to the outer leaflet (see Section 2.2.2). Thus, phospholipid asymmetry results from the balance of the active translocation of phosphatidylserine and phosphatidylethanolamine and the passive slow bidirectional flip/flop of phospholipids.

Red cell lipids exist in different domains within each of the bilayer planes (see Section 2.2.1); that is a *cis* asymmetry, related to macroscopic and microscopic domains of the membrane lipids. These lipid-rich domains are intrinsic structural features of the membrane. Lipids also partition on a microscopic scale within the membrane. Positively charged amino acids are concentrated on the cytoplasmic side of the bilayer-spanning domains of glycoporphins and other membrane proteins, because glycoporphin A binds anionic (phosphatidylserine and phosphatidylinositol) but not choline (phosphatidylcholine and sphingomyelin) phospholipids. Anionic phospholipids appear to cluster near the regions of positive charge.

The red cell shape is also dependent on the conditions of membrane lipids (see Section 2.2.6). Processes that expand the outer bilayer or contract the inner bilayer will produce uniform membrane spiculation (echinocytes), whereas relative expansion of the inner leaflet will lead to membrane invagination and cup-shaped red cells (stomatocytes). Strongly charged amphipathic compounds, such as phospholipids, cause echinocytes. Phospholipids are trapped in the outer bilayer by their fixed charge. Permeable amphipathic compounds will cause the membrane to extend toward the side of greater accumulation, because they are weak acids and bases that can cross the membrane in their uncharged form. Cationic compounds tend to accumulate in the negatively charged inner bilayer and anionic compounds partition to the neutral outer bilayer. The bilayer couple hypothesis predicts that shape changes resulting from expansion of one lipid leaflet can be reversed by a commensurate alteration in the other, as seen in the intensely spiculated acanthocytosis in patients with abetalipoproteinemia, which can be almost completely converted into normal biconcave disc shapes by the addition of a cationic amphipathic compound (0.1 mM chlorpromazine).

Cholesterol and glycolipids are intercalated between the phospholipids in the bilayer within their long axes perpendicular to the bilayer plane (see Section 3.1). Cholesterol is present in about equal proportions on both sides of the bilayer, and equilibrates between them in seconds, or less. Cholesterol depletion promotes inward curvature of the membrane, whereas cholesterol enrichment favors outward deflection.

Mature red cells cannot synthesize fatty acids, phospholipids, or cholesterol *de novo* and depend on lipid exchange and fatty acid acylation as the mechanisms for phospholipid renewal and repair (see Section 2.2.4). Phosphatidylcholine and sphingomyelin, which are outer bilayer phospholipids, exchange slowly with the

phospholipids of plasma lipoproteins. Inner bilayer phospholipids (phosphatidylserine and phosphatidylethanolamine) are basically unexchangeable. Thus, the abnormalities of plasma lipoproteins induce serious effects on the red cell membranes, as observed in abetalipoproteinemia [3] and Tangier disease [4], which are described in detail later (see Chapter 17). In contrast, free unesterified cholesterol in red cell membranes are able to exchange readily with the unesterified cholesterol in plasma lipoproteins ($T_{1/2}$ of 7 h), where it is partially converted into esterified cholesterol by the action of a plasma enzyme, lecithin: cholesterol acyltransferase (LCAT) (see Section 2.2.4). LCAT catalyzes a unidirectional pathway that depletes the membrane of cholesterol and decreases its surface area, because the newly formed cholesteryl ester cannot return to the red cell membrane. When LCAT is absent, excess membrane cholesterol accumulates, expanding the membrane surface area. A detailed description of a case of LCAT deficiency [5] will be given in the following section (see Section 17.2).

From the functional standpoint, lipid mobility is crucial in red cell membrane physiology (see Section 2.2.3). Purified phospholipids exhibit discrete, liquid crystalline to gel phase transitions that are dependent on the length and degree of unsaturation of their acyl side chains. Above this transition temperature, the acyl side chains can move very quickly. Below the transition temperature to the gel phase, acyl chains of purified lipids are extended in stiff, parallel, hexagonally packed arrays that are more like a solid than a liquid. The extent of membrane lipid fluidity is dependent on several factors (see Section 2.2.3), that is: the type of cholesterol (free or esterified), the class of phospholipids, the molar ratio of cholesterol to phospholipids, the degree of saturation of fatty acids, the length of acyl chains, and the presence or absence of amphipathic compounds such as lysophosphatides. Compensation for an alteration in one or more of these variables to give normal membrane fluidity is found to be present in nature, which is known as homeoviscous adaptation. Membrane fluidity in red cell membranes has been studied extensively by the method of electron spin resonance (ESR) in various disease states (see Sections 17.2, 17.3 and 17.4), such as familial abetalipoproteinemia, congenital lecithin:cholesterol acyltransferase deficiency, and hereditary high red cell membrane phosphatidylcholine hemolytic anemia. Detailed results on these disorders are shown in each section.

The incidence of red cell membrane lipid disorders of hereditary origin appears to be extremely low, although a detailed statistical survey has not been carried out. In our experience at Kawasaki Medical School as the Japanese government-assigned reference institution for red cell membrane disorders in Japan, membrane lipid abnormalities of hereditary origin were found in 41 patients (4.0 %) from 27 kindred out of our 1014 cases of hereditary red cell membrane disorders from 605 kindred in the Japanese population (Table 9.1). Among these red cell membrane lipid abnormalities, we experienced 31 patients of hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCA) from 19 kindred, eight cases from six kindred of congenital β -lipoprotein deficiency (acanthocytosis), one patient with congenital lecithin:cholesterol acyltransferase deficiency, and one patient with congenital α -lipoprotein deficiency (Tangier disease). Contrary

to the abnormalities of plasma factors, such as lipoproteins and enzyme, which are observed in β -lipoprotein deficiency, α -lipoprotein deficiency, and lecithin:cholesterol acyltransferase deficiency, the exact pathogenesis of hereditary high red cell membrane phosphatidylcholine hemolytic anemia has not been elucidated (see Section 17.4). In this disorder, red cell membrane lipid composition is clearly abnormal, although plasma lipids appear to be unaffected. Detailed descriptions are presented in the following sections.

There are also numerous acquired cases with red cell membrane lipid abnormalities due to plasma lipid anomalies through hepatic dysfunctions (see Sections 13.5 and 17.6). Typical disorders are spur cell anemia and target cells, which are also discussed in the following sections.

17.2

Lecithin: Cholesterol Acyltransferase (LCAT) Deficiency

A plasma enzyme, lecithin:cholesterol acyltransferase (LCAT) [6], mediates the esterification of plasma cholesterol by a mechanism that involves the transfer of fatty acids from phosphatidylcholine (PC) to free cholesterol (FC), generating cholesteryl esters (CEs) and lysophosphatidylcholine (L-PC) [7]. A major role for this enzyme lies in the reverse cholesterol transport, by which cholesterol is transferred from peripheral cells to the liver for catabolism. The process in detail [5] is that free cholesterol in the cells is taken up by high density lipoprotein (HDL) and is esterified by LCAT [8]. The cholesterol esterified by this process is packed in the core of the lipoprotein, resulting in the maturation of discoidal pre- β -HDL to spherical α -HDL. Following this event, the esterified cholesterol may be exchanged for very low density lipoprotein (VLDL) triglycerides by the cholesteryl ester transfer protein (CETP) for transport back to the liver, or may be taken up directly by the liver [9].

LCAT in human beings is synthesized mainly by the liver as a 416 amino acid glycoprotein of approximately 63 kDa [10]. LCAT resides in plasma reversibly bound chiefly to HDLs, which contain its major activator, apolipoprotein (apo) A-I [11]. Human LCAT prefers to transfer an unsaturated sn -2 chain from PC to cholesterol [12]. The primary structure of LCAT [13] is highly conserved among various species, indicating that the full intact protein is required for enzymatic activity. Structurally, LCAT is a complex enzyme consisting of multiple functional domains that are required for LCAT activity [14]. These include a large active site (serine at 181, aspartic acid at 345, and histidine at 377), which is capable of binding phospholipids and esterified cholesterol simultaneously. A lipid-binding lid region, encompassing residues 50 to 74, covers the active site that may participate in interfacial activation. In the enzyme molecule, a lipoprotein-binding domain (residues 130 to 306) may play a role in apo A-I binding as well as modulation of LCAT substrate specificity [15, 16].

The human LCAT gene is located in the 16q22.1 region [17]. This gene has six exons that encode the 440 amino acids including 24 signal peptide residues, that comprise the LCAT protein [13]. Although LCAT is expressed primarily in the

liver, its mRNA also can be detected at much lower levels in the brain and testes of mice, rabbit, and nonhuman primates. Alternative splicing resulting in the insertion of an Alu cassette between exons 5 and 6 has been reported for both human and nonhuman primate mRNA [18]. Regarding the regulation of LCAT gene expression, LCAT gene expression is relatively resistant to dietary challenges and drug treatments [5].

Lipoprotein metabolism can be divided into two major pathways, that is, the apo B-containing lipoprotein (apo B-Lp) and HDL pathways [5]. In the process of reverse cholesterol transport, the cholesterol in lipoproteins is transported to the liver as free cholesterol and cholesteryl esters [5]. Both HDL and apo B-Lp each transport approximately 50 % of the total cholesterol to the liver. In human beings, roughly 90 % of the free cholesterol is transported to the liver by HDL, and more than 90 % of the cholesteryl esters is transported by the apo B-Lps [19]. A large fraction of the cholesteryl esters in the apo B-Lps transported to the liver is derived from HDL through the action of cholesteryl ester transfer protein.

The first case of autosomally inherited familial LCAT deficiency was discovered in a Norwegian family with corneal opacities, anemia, proteinuria, mild hypoalbuminemia, and hyperlipidemia [20]. Plasma cholesterol, triglyceride, and phospholipid levels were increased, but cholesteryl esters and lysophosphatidylcholine were decreased and HDL (pre- β and α -lipoproteins) could not be detected [21, 22]. There was a total lack of LCAT activity in plasma due to a missense mutation (ATG→AAG at codon 252: methionine→lysine) of the LCAT gene [23]. Up to the present time, at least 29 different mutations of the LCAT gene have been reported (Table 17.1), among which 20 missense mutations, seven frameshift mutations and two nonsense mutations have been described [5]. The affected individuals are homozygotes of the mutations (21 cases) or compound heterozygotes (14 cases). Most mutations in the LCAT gene exhibit enzymatic activity that is almost missing in the range from 0 to 50 % of normal, indicating that the entire molecule of LCAT is required for normal structural integrity and its function [5]. The location of these mutations is widely distributed over whole the LCAT gene [5].

Hematological characteristics of the familial LCAT deficiency are mild normocytic normochromic anemia with mild reticulocytosis and target cells [24]. The red cell life span is reduced to one half that of normal individuals. The presence of foam cells (sea-blue histiocytes) can be observed in bone marrow specimens. The histiocytic granules are composed of membranes in a lamellar arrangement. The patient's red cell membranes exhibit abnormal lipid content; that is, a two-fold increase in unesterified cholesterol and phosphatidylcholine with decreased sphingomyelin and phosphatidylethanolamine [25]. A marked reduction in both acetylcholinesterase activity and sodium influx has been reported [26].

A mouse model for human LCAT deficiency has been generated by targeted disruption of the LCAT gene [27, 28]. The activity of α -LCAT was virtually missing (0.75 % of normal). Homozygous LCAT-deficient mice have reduced total cholesterol (23 % of normal), HDL-cholesterol (7 % of normal), and apo A-I (14 % of normal) and increased triglycerides in plasma (212 % of a normal control). No evidence of corneal opacities was detected in the mutated mice [27, 28].

Table 17.1 A list of gene mutations in congenital lecithin: cholesterol acyltransferase (LCAT) deficiency.

Location (Exon)	Nucleotide	Protein	Genotype	LCAT activity (%)
1	A→T	5 Asn→Ile	Homo	18
1	30 bp insertion	7 Leu	CH	4
1	C insertion	9 Pro	Homo	0–9
2	G→A	30 Gly→Ser	Homo	0
2	T→C	32 Leu→Pro	CH	<10
2	C→A	33 Gly→Arg	CH	4
3	C→A	83 Tyr→Stop	Homo	7
3	C→A	83 Tyr→Stop	CH	2
3	G→A	93 Ala→Thr	Homo	5
4	AC→T deletion	120 Tyr	CH	2
4	C→T	135 Arg→Trp	CH	0
4	G→A	140 Arg→His	Homo	0
4	GGC insertion	141 Gly	Homo	2
4	C→T	147 Arg→Trp	Homo	0
4	C→T	147 Arg→Trp	CH	2
5	T→A	156 Tyr→Asn	CH	2
5	C→T	158 Arg→Cys	Homo	5
5	C deletion	168 His	CH	0
5	T→G	171 Tyr→Stop	CH	2
5	G→A	183 Gly→Ser	CH	2
5	T→C	209 Leu→Pro	Homo	0–15
6	C→A	228 Asn→Lys	Homo	0
6	G→A	234 Met→Ile	Homo	50
6	C→G	244 Arg→Gly	Homo	12
6	C→G	244 Arg→Gly	Homo	13
6	T→A	252 Met→Lys	Homo	0
6	G deletion	264 Val	Homo	0
6	G→A	293 Met→Ile	Homo	12
6	G→A	293 Met→Ile	Homo	8–9
6	C→T	321 Thr→Met	Homo	5
6	C→T	321 Thr→Met	CH	0–10
6	G→A	344 Gly→Ser	Homo	0
6	A insertion	376 Gln	CH	0
6	C→T	399 Arg→Cys	Homo	17
6	C→T	399 Arg→Cys	CH	2

Homo: homozygotes, CH: compound heterozygotes.

Deficiency of the plasma enzyme, lecithin:cholesterol acyltransferase is known to produce marked alterations in plasma lipids, particularly increased free cholesterol. The membrane lipids in mature red cells are dependent on the plasma lipids because of the lack of *de novo* synthesis of the membrane lipids (see Section 2.2.4). Thus, the marked abnormality of plasma lipids in lecithin:cholesterol acyltransferase deficiency should affect the red-cell membrane lipids. However, it is also shown that the increase in red-cell membrane fluidity in lecithin:cholesterol acyltransferase deficiency is surprisingly small in view of the extensive alterations both in the membrane lipid composition and in the functional properties of these cells. Free cholesterol is known as a hardening effector in red-cell membrane fluidity (see Section 2.2.3), and the decreased membrane fluidity may lead the abnormal red cells to early destruction, through, for instance, increased hemolysis. Thus, the compensation mechanism against increased free cholesterol is interesting to investigate both in the plasma and in the red cells in cases of lecithin:cholesterol acyltransferase deficiency. For this purpose, the classes of lipid, the fatty acid composition, the length of the chains and the saturation of the double bonds were analyzed in the plasma lipids and the red cell membrane lipids [25]. The membrane fluidity of the patient's red cells was also determined in the intact red cells, in the liposomes of total lipids, and in cholesterol-depleted liposomes.

The patient (a 49 year old Japanese male) was found to have compensated hemolysis, hepatosplenomegaly without liver dysfunctions, and a marked poikilocytosis in red-cell morphology (Fig. 17.1) [25]. The activities of plasma enzyme, lecithin:cholesterol acyltransferase (nmol mL^{-1} per hour) in the patient were clearly deficient in determinations both by the method of Glomset and Wright (9.6 ± 11.1 : control 93.3 ± 16.8) and by that of Stokke and Norum (2.3 ± 1.4 : control 66.3 ± 11.1) [20]. The red cell membrane studies revealed a normal composition of membrane proteins on sodium dodecylsulfate polyacrylamide gel disc electrophoresis, and decreased sodium influx (0.93 mmol L^{-1} red cells per hour: control 1.49 ± 0.14) [26].

Free cholesterol was markedly elevated in the patient's plasma, contrary to the significant decrease of the esterified form due to the deficiency of lecithin:cholesterol acyltransferase activity (Table 17.2) [25]. High-density lipoprotein (HDL) cholesterol was also diminished considerably. Among the plasma phospholipids,

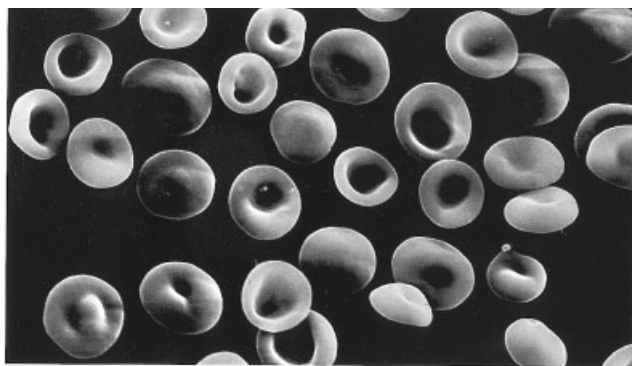


Figure 17.1 Scanning electron micrograph of red cells of a patient with congenital lecithin:cholesterol acyltransferase deficiency.

sphingomyelin was decreased, and PC was slightly increased [25]. Lipoprotein X was detected on agar gel electrophoresis, in addition to an increase in triacylglycerols.

Table 17.2 Plasma lipids and red cell lipids in the patient with congenital lecithin: cholesterol acyltransferase (LCAT) deficiency.

I. Plasma lipids		
	Patient (n = 4)	Normal (n = 5)
Cholesterol (mg dL ⁻¹)		
Total	194 ± 28	178 ± 32
Free	176 ± 26 (90.6 ± 1.3%)*	49 ± 9 (27.5 ± 5.0%)
Esterified	18 ± 3 (9.4 ± 0.2%)*	129 ± 23 (72.5 ± 12.9%)
High-density-lipoprotein cholesterol (mg dL ⁻¹)	3 ± 1*	50 ± 11
Phospholipids (%)		
Lysophosphatidylcholine	0.1 ± 0.1	4.8 ± 2.4
Phosphatidylcholine	77.9 ± 4.8	69.2 ± 6.3
Sphingomyelin	8.4 ± 1.2**	17.2 ± 2.7
Phosphatidylethanolamine	7.1 ± 2.1	3.4 ± 3.1
Phosphatidylserine + phosphatidylinositol	5.0 ± 2.1	2.3 ± 2.3
Others	1.5 ± 0.9	2.2 ± 2.6
Triacylglycerols (mg dL ⁻¹)	284 ± 39**	95 ± 65
Lipoprotein X	present	—

*Significant in $P < 0.001$. ** $P < 0.01$.

II. Red cell lipids		
	Patient (n = 3)	Normal (n = 5)
Free cholesterol (FC)	1763*	1202 ± 103
Total phospholipids (PL)	3435*	2604 ± 241
Lysophosphatidylcholine (L-PC)	21 (0.6)	34 ± 18 (1.3 ± 0.7)
Phosphatidylcholine (PC)	1738 (50.6)*	747 ± 73 (28.7 ± 2.8)
Sphingomyelin (SM)	546 (15.9)*	674 ± 49 (25.9 ± 1.9)
Phosphatidylethanolamine (PE)	687 (20.0)*	805 ± 42 (30.9 ± 1.6)
Phosphatidylserine (PS) + phosphatidylinositol (PE)	443 (12.9)	344 ± 34 (13.2 ± 1.3)
(PC + SM + L-PC)/(PE + PS + PI)	2.04	1.27 ± 0.04
FC/PL ratio	1.00	0.90 ± 0.04

* Significant in $P < 0.01$. Values indicate μg per 10^{10} red blood cells. Numbers in parentheses represent percentage of phospholipids.

A distinct abnormality was noted in red cell membrane lipids of the patient, particularly the elevation of free cholesterol, associated with a profound increase in PC (Table 17.2) [25]. In contrast, the level of sphingomyelin and the PE content were significantly decreased. The ratio of free cholesterol to total phospholipids was maintained normally, in spite of these marked abnormalities of red cell membrane lipids [25]. Increased free cholesterol in the patient's red cells was counteracted chiefly by the increased PC.

In the fatty acid composition of total plasma lipids, a shorter chain 16:0 was increased, and unsaturated fatty acids, such as 18:1, were also increased (Table 17.3) [25]. For PC extracted from the patient's plasma, an increase in a shorter chain 16:0 and a decrease in a longer chain 18:0 were observed. In the case of sphingomyelin, 16:0 was increased, concomitant with a decrease in 22:0 and 24:0. The 16:0 was also increased in PE. In the case of total red cell lipids, a shorter chain 16:0 was increased, and longer chains 18:0 and 24:0 were somewhat decreased. Unsaturated fatty acids, such as 18:2 and 20:5, were increased. The overall unsaturation index (160.8: control 152.1) was significantly increased. In PC, 16:0 was also increased, contrary to the decrease in 18:0 as a longer acyl chain. The unsaturation index (115.0: control 106.2) was also increased. In the cases of PE, sphingomyelin and phosphatidylserine + phosphatidylinositol, no major changes were observed, except for a slight increase in the polyunsaturated chains in the PE and phosphatidylserine fractions [25].

Analysis of electron spin resonance spectra of spin-labeled phospholipids extracted from the red cells of the patient was carried out (Table 17.4) [25].

For the liposomes of total lipids, the liposomes free of cholesterol prepared from the red cell membranes, and intact red cells of normal subjects and of the patient, the parameter, *S*, was calculated from results of the ESR spectra. In general, the increased free cholesterol content, as observed in the patient, decreases the membrane fluidity, whereas the concomitantly increased PC content enhances red cell membrane fluidity [25].

In the phospholipid liposomes free of cholesterol, order parameters in ESR were decreased over the lower temperature range. In contrast to these results, total lipid liposomes, including cholesterol, showed no abnormalities over a wide range of temperatures. Thus, the cholesterol-depleted phospholipid liposomes appear to possess increased fluidity [25].

For the intact red cells, the order parameters of ESR spectra in the patient were normal at various temperatures, as with the results for the total lipids with enhanced cholesterol. Thus, membrane proteins appeared not to affect the order parameters in ESR spectra.

The membrane fluidity (see Section 2.2.3) is known to be dependent on the type of cholesterol (free or esterified), the classes of phospholipid, the molar ratio of cholesterol to phospholipids, the saturation of the double bonds, the number of carbons in the acyl chains, and the presence or absence of amphipathic compounds such as lysophosphatides.

Free cholesterol decreases the membrane lipid fluidity, while esterified cholesterol increases it. PC increases membrane fluidity, and sphingomyelin and PE de-

Table 17.3 Fatty acid composition of plasma lipids and red cell lipids of the patient with congenital lecithin: cholesterol acyltransferase (LCAT) deficiency.

I. Plasma lipids

	Total lipids			Phosphatidylcholine (PC)		Sphingomyelin (SM)		Phosphatidylethanolamine (PE)	
	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)		Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)
DMA	—	—	—	—	—	—	—	21.9	5.7 ± 1.1
16 : 0	25.9*	21.0 ± 1.1	34.0*	—	28.9 ± 1.1	37.4*	—	17.4*	14.4 ± 1.4
16 : 1	2.4	1.6 ± 0.9	—	—	—	—	—	—	—
DMA	—	—	—	—	—	—	—	3.3	5.7 ± 0.7
18 : 0	9.9	7.5 ± 0.4	12.8*	—	14.5 ± 0.3	8.5	—	17.4*	13.9 ± 1.2
18 : 1	29.2*	18.6 ± 2.3	13.9	—	10.5 ± 0.5	5.4	—	17.0*	10.2 ± 1.4
18 : 2	18.2*	33.5 ± 4.5	26.1	—	26.1 ± 2.4	6.1	—	15.8*	19.0 ± 3.2
20 : 0	—	—	—	—	—	2.8*	—	—	—
20 : 1	—	—	—	—	—	—	—	—	—
20 : 3	0.4	1.0 ± 0.3	1.0	—	2.2 ± 0.5	—	—	—	—
20 : 4	3.1*	5.7 ± 0.6	4.1*	—	7.1 ± 0.7	—	—	9.4	11.2 ± 1.7
20 : 5	1.8	1.7 ± 0.6	2.7	—	2.4 ± 0.6	—	—	4.6*	2.4 ± 0.7
22 : 0	—	—	—	—	—	6.2*	—	—	—
22 : 1	—	—	—	—	—	1.3	—	—	—
22 : 2	—	—	—	—	—	2.4	—	—	—
22 : 5	—	—	—	—	—	1.2	—	0.9	1.1 ± 0.3
22 : 6	3.2	4.9 ± 0.7	3.8*	—	5.3 ± 0.5	—	—	9.8	9.7 ± 1.7
24 : 0	0.3	0.5 ± 0.1	—	—	—	4.2*	—	—	—
24 : 1	0.6	0.9 ± 0.1	—	—	—	21.7	—	—	—

* Significant in *P* < 0.01.

II. Red cell lipids

Total lipids			Phosphatidylcholine (PC)		Sphingomyelin (SM)		Phosphatidylethanolamine (PE)		Phosphatidylserine (PS)	
	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)	Lecithin: cholesterol acyltransferase deficiency (n = 3)	Normal (n = 5)
DMA	—	—	—	—	—	—	6.0*	4.8 ± 0.3	—	—
16 : 0	26.8*	21.8 ± 1.2	41.7*	34.9 ± 0.8	24.1**	26.2 ± 1.1	15.3	16.0 ± 0.5	2.8	3.8 ± 0.7
DMA	—	—	—	—	—	—	9.1	8.0 ± 0.9	—	—
18 : 0	12.5*	14.3 ± 0.4	7.7*	12.2 ± 0.4	5.7*	7.1 ± 0.5	9.9*	8.7 ± 0.2	48.4	46.7 ± 2.8
18 : 1	11.2*	13.4 ± 1.0	14.0*	17.2 ± 0.6	4.2*	2.4 ± 0.6	11.3*	17.0 ± 0.8	4.8*	8.5 ± 0.7
18 : 2	15.3*	10.8 ± 1.0	24.3**	22.6 ± 1.0	4.8*	2.2 ± 0.8	5.8	6.6 ± 1.2	2.4	2.3 ± 0.2
20 : 4	11.1	12.0 ± 0.8	4.8	4.6 ± 0.6	—	—	17.1	16.4 ± 0.9	15.5*	18.2 ± 0.7
20 : 5	3.1*	1.5 ± 0.5	2.8*	1.6 ± 0.4	—	—	5.5*	3.0 ± 0.7	1.1	0.9 ± 0.4
22 : 0	—	—	—	—	6.0*	7.0 ± 0.4	—	—	—	—
22 : 4	—	—	—	—	—	—	2.8	3.4 ± 0.5	1.9	2.0 ± 0.3
22 : 5	2.2**	2.8 ± 0.3	—	—	2.7*	4.4 ± 0.5	4.1	3.8 ± 0.4	4.3*	3.2 ± 0.4
22 : 6	7.6	7.2 ± 0.9	3.2	2.9 ± 0.4	—	—	11.7*	9.5 ± 0.9	16.1*	10.8 ± 0.9
24 : 0	2.7*	4.9 ± 0.7	—	—	21.3	19.1 ± 0.8	—	—	—	—
24 : 1	2.5*	4.4 ± 0.9	—	—	26.3	26.6 ± 1.5	—	—	—	—

* Significant in $P < 0.01$. ** Significant in $P < 0.02$.
 Values are percentages. DMA, dimethylacetal derivatives.

Table 17.4 Membrane fluidity by electron spin resonance (ESR) evaluated by order parameters for intact red cells, and liposomes of extracted membrane lipids of the patient with congenital lecithin: cholesterol acyltransferase (LCAT) deficiency.

I. Intact red cells

	<i>Temperature (°C)</i>	<i>2T (Parallel) (G)</i>	<i>2T (Perpendicular) (G)</i>	<i>a' (G)</i>	<i>S</i>
Lecithin:	47	52.5	19.0	15.1	0.613
cholesterol	45	52.8	18.8	15.1	0.623
acyltransferase	43	53.0	18.6	15.0	0.631
deficiency	41	53.4	18.5	15.1	0.639
	39	53.8	18.4	15.1	0.647
	37	54.1	18.1	15.1	0.660
	35	54.3	18.2	15.1	0.659
	33	54.9	18.0	15.2	0.672
	31	55.5	17.9	15.2	0.682
	29	56.2	17.7	15.3	0.696
	27	56.5	17.6	15.3	0.702
Control	47	52.6	18.8	15.0	0.620
	45	52.9	18.7	15.1	0.627
	43	53.6	18.5	15.1	0.641
	41	53.8	18.4	15.1	0.647
	39	54.1	18.3	15.1	0.653
	37	54.1	18.2	15.1	0.657
	35	54.8	18.1	15.2	0.668
	33	55.4	17.9	15.2	0.681
	31	56.0	17.7	15.2	0.694
	29	56.3	17.6	15.3	0.700
	27	57.4	17.5	15.4	0.715
	25	57.7	17.3	15.4	0.725

Determinations in triplicate.

II. Liposomes of extracted membrane lipids

	<i>25 °C</i>	<i>30 °C</i>	<i>37 °C</i>
Phospholipids (Cholesterol minus)			
Normal 1	0.655	0.624	0.592
Normal 2	0.660	0.626	0.592
LCAT deficiency	0.639	0.609	0.582
Total lipids			
Normal 1	0.705	0.676	0.643
Normal 2	0.707	0.679	0.648
LCAT deficiency	0.697	0.670	0.644

In two determinations in two each specimen.

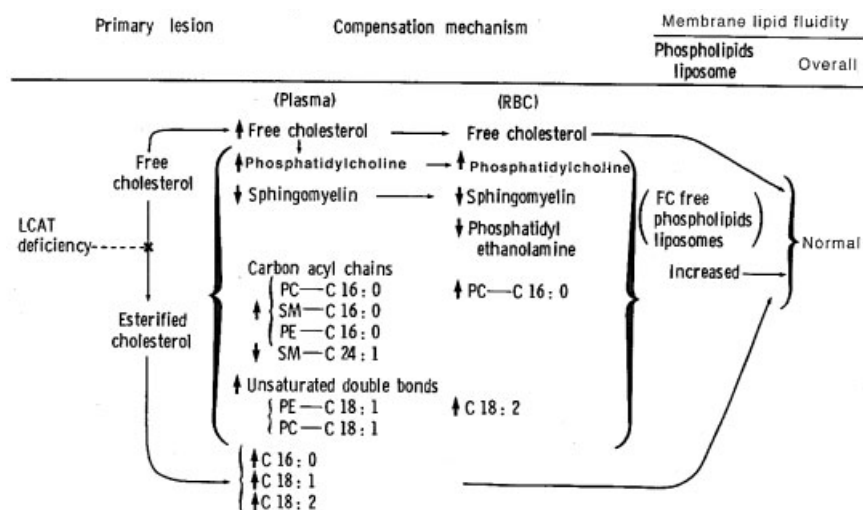


Figure 17.2 Schematic diagram of a compensation mechanism of red cell membrane lipid fluidity by altering lipid composition in congenital lecithin:cholesterol acyltransferase deficiency.

crease it. The shorter acyl chain length and the increased unsaturated fatty acids of these phospholipids enhance the membrane fluidity, in contrast to the decrease in membrane lipid fluidity, through the presence of the longer acyl chain length and by increasing the saturation of the fatty acids. Lysophosphatides are known to increase the fluidity.

The results were summarized as follows (Fig. 17.2).

Owing to the deficiency of the plasma enzyme, lecithin:cholesterol acyltransferase, free cholesterol accumulated in the patient's plasma. Thus, the increased free cholesterol decreases the membrane lipid fluidity substantially.

It is interesting to note that, counteracting the higher concentration of free cholesterol, PC was markedly increased, which may ease the decreased fluidity caused by free cholesterol. Sphingomyelin in the patient's plasma was decreased which will also alleviate the decreased fluidity. Shorter carbon acyl chains (such as 16:0) were increased in the total plasma lipids, PC, sphingomyelin and PE, and longer carbon chains were decreased (such as 22:0 and 24:0 in sphingomyelin). Unsaturated double bonds (such as 18:1) were increased in PE and PC.

These changes affect red cell membrane lipids directly. In red cells, free cholesterol was markedly increased, which decreased the membrane fluidity. However, the concomitant increase in PC leads to an increase in the fluidity, and a decrease in sphingomyelin and in PE also tends to increase the fluidity. The 16:0 was increased in total lipids and in PC in the red cell membrane lipids, which may soften the membranes. Unsaturated double bonds, such as 18:2, were also increased, as a softening effector.

Finally, membrane lipid fluidity was investigated using red cell membrane liposomes free of cholesterol. The order parameters in ESR showed increased fluidity in the membrane liposomes when free cholesterol was omitted [25].

In the same way, the total lipid liposomes including free cholesterol were investigated with ESR. The order parameter was completely normal [25]. The ESR spin label studies on the intact red cells also revealed normal membrane fluidity. The results can be interpreted as follows. The increased free cholesterol, which is the primary lesion of this disorder, decreased the membrane lipid fluidity. In contrast to this, phospholipids were changed in nature, counteracting the increased FC. Thus, the overall membrane lipid fluidity was, surprisingly, maintained normally [25].

These results may suggest the presence of a finer self-adaptive control mechanism counteracting the lipid abnormalities induced by a hereditary metabolic defect [29].

The ESR spin-label studies were also performed in intact red cells, in addition to the extracted membrane lipids, as mentioned above, in which membrane proteins were stripped from the whole intact red cells. The results on intact red cells showed normal membrane fluidity in the patient. Thus, the increased free cholesterol was also counteracted by self-adaptive softening effectors, which keep the patient's red cell membrane fluidity normal.

Finally, since Ca^{2+} has pronounced effects on membrane fluidity, the possibility exists that divalent cations, especially Ca^{2+} , may be involved in the self-adaptive mechanism, and not merely lipid and fatty acid alterations. Thus, calcium contents in plasma and in red cells were determined in 54 normal subjects and in patients with lecithin:cholesterol acyltransferase deficiency. There were no significant differences in plasma calcium between normal subjects ($103 \pm 39 \mu\text{g mL}^{-1}$) and those with lecithin:cholesterol acyltransferase deficiency (98 ± 29), and in red cell calcium between normal ($1.02 \pm 0.61 \mu\text{g mL}^{-1}$ red cells) and lecithin:cholesterol acyltransferase deficiency (1.13 ± 0.49), respectively. These results indicate that the effects of Ca^{2+} are unimportant, even in the intact red cells.

These results will help in our understanding of the physiological homeoviscous adaptation mechanism [29] in the membrane lipids of patients with altered lipid metabolism.

17.3

β -Lipoprotein Deficiency (Acanthocytosis)

Abetalipoproteinemia is characterized by an association of acanthocytosis (Fig. 17.3) with atypical retinitis pigmentosa, progressive ataxic neurologic disease, and a celiac disease-like lipid malabsorption [3]. This disorder with autosomal recessive inheritance was first described by Bassen and Kornzweig in 1950 [30]. There are no examples of an affected parent and child. Subsequently, preparative ultracentrifugation confirmed the virtual absence of low density lipoproteins (LDL) in patients with homozygous abetalipoproteinemia. The malabsorption involves triglycerides, with a defect at the level of the intestinal epithelium. The absence of β -lipoproteins is a unifying feature and the first clue to the underlying mechanisms.



Figure 17.3 Scanning electron micrograph of red cells in congenital β -lipoprotein deficiency (acanthocytosis).

In normal human beings, very low density lipoproteins (VLDL) and chylomicrons, which transport triglycerides to peripheral tissues through peripheral blood, are major lipoprotein secretory products of the liver and intestine, respectively [5]. Each class of these lipoproteins contains a protein of very high molecular weight (a B apolipoprotein) that is essential for the secretion of the lipoprotein particle and that has a very high affinity for lipids. There are two translation products of a single structural gene for B apolipoproteins [31]. The B apolipoprotein of VLDL and LDL is apo B-100, which is a single polypeptide chain of 4536 amino acid residues and the full-length translation product of the gene [32]. The main species of apo B in chylomicrons is apo B-48, which is a single chain of 2152 amino acid residues identical to the N-terminal portion of the full-sized apo B-100 [33].

In abetalipoproteinemia, chylomicrons and all lipoproteins that contain B apolipoproteins are absent from blood [34]. The levels of cholesterol and triglycerides are markedly reduced, because these lipoproteins carry most of the cholesterol and triglycerides in plasma. The content of exogenous polyunsaturated fatty acids such as linoleate is decreased in plasma and adipose tissue, indicating inefficient uptake of dietary fat [34]. The relative content of phosphatidylcholine is reduced and that of sphingomyelin is increased.

We have experienced eight patients from six kindred of abetalipoproteinemia out of 1014 cases from 605 kindred with hereditary red cell membrane disorders in the Japanese population [35]. The analyses of plasma lipids in one of these patients [35] revealed levels of total cholesterol of 31 mg dL^{-1} (normal: 189 ± 35), free cholesterol of 11 mg dL^{-1} (normal: 49 ± 9), triglycerides of 16 mg dL^{-1} (normal: 104 ± 42), total phospholipids of 32 mg dL^{-1} (normal: 173 ± 29), free fatty acids of 66 mEq L^{-1} (normal: 450 ± 150), and β -lipoproteins of 3 mg dL^{-1} (normal: 350 ± 150), indicating striking abnormalities of the plasma lipids. Abnormal plasma lipids in other two patients of this disorder are also shown in Table 17.5. In addition to these results, the fractions of apolipoproteins were also decreased, that is: apolipoprotein A-I of 48.4 mg dL^{-1} (normal: 115 ± 15), apolipoprotein A-II of 10.9 mg dL^{-1} (normal: 33 ± 6), apolipoprotein E of 3 mg dL^{-1} (normal: 4.3 ± 1.1), apolipoprotein C-II only in a trace amount (normal: $4 \pm 1.2 \text{ mg dL}^{-1}$), and finally no detectable apolipoproteins (B-100, and B-48; normal: $85 \pm 12 \text{ mg dL}^{-1}$).

Table 17.5 Plasma lipids and red cell lipids in the patients with congenital β -lipoprotein deficiency (acanthocytosis).**I. Plasma lipids**

<i>Plasma lipids (mg dL⁻¹)</i>	<i>Patients</i>		<i>Normal subjects</i>
	<i>1</i>	<i>2</i>	
Total cholesterol	20	23	178 $\pm$ 32
Free cholesterol	16	16	49 $\pm$ 9
Esterified cholesterol	4	4	129 $\pm$ 20
Total phospholipids	25	27	75 $\pm$ 8
Phosphatidylcholine (%)	47.6	50.4	69.2 $\pm$ 6.3
Sphingomyelin (%)	37.5	35.7	17.2 $\pm$ 2.7

II. Red cell lipids

<i>Red cell lipids (μg per 10¹⁰ red cells)</i>	<i>Patients</i>		<i>Normal subjects</i>
	<i>1</i>	<i>2</i>	
Free cholesterol	1364	1514	1204 $\pm$ 103
Total phospholipids	2398	2591	2604 $\pm$ 241
Sphingomyelin	643	731	663 $\pm$ 73
Phosphatidylcholine	493	565	733 $\pm$ 64
Lyso-phosphatidylcholine	20	23	39 $\pm$ 11
Phosphatidylserine	453	479	366 $\pm$ 38
Phosphatidylethanolamine	789	793	806 $\pm$ 86

The lipoproteins of the LDL exhibit a decreased ratio of lecithin to sphingomyelin (almost 1 in contrast to a normal ratio of 2). Nearly half the cholesterol is unesterified as free cholesterol, compared with less than one-third in normal LDL. The HDL have an abnormally high ratio of free to esterified cholesterol (0.7 compared with 0.3) and a lecithin:sphingomyelin ratio of about 5:4 (compared with a normal ratio of approximately 8:1). The levels of vitamin A, and vitamin D were 224 IU (normal: 410–1200), and 10.7 ng mL⁻¹ (normal: 10–30). The content of vitamin E in this patient was undetectable (normal: 5–20).

Sensitive detection methods reveal the presence of small amounts of apo B of normal molecular weight including a complex with apo (a) [36], or at least of truncated molecules that include the N-terminus consistent with the concept that failure to lipidate apo B 100 results in cotranslational proteolysis [37].

A number of studies provided several pieces of important evidence, that is: (1) the apolipoproteins that appear in plasma in abetalipoproteinemia all appear to be structurally normal, (2) the apo B gene is basically not involved, (3) the apo B detected in liver and intestine in this disorder includes a full-length product, (4)

the quantities of B proteins in liver and intestine are less than in normal tissues, but levels of apo B message are greatly increased, and (5) abetalipoproteinemia, in most cases, is probably due to defects of one or more proteins involved in processing apo B through the secretory pathway for VLDL and chylomicrons.

As a cause of abetalipoproteinemia, defects in the microsomal triglyceride transfer protein (MTP) are first suggested by the absence of that protein from intestinal and hepatic microsomes in affected homozygotes [38]. MTP is known to catalyze the transport of triglycerides, cholesterol ester, and phospholipids from phospholipid surfaces, and to be required for secretion of apolipoprotein B containing lipoproteins [3, 39–41]. When the MTP gene is mutated, apoprotein B becomes absent in plasma, along with each lipoprotein fraction that contains this apoprotein. These lipoprotein fractions include chylomicrons and VLDL, which transport triglycerides, and LDL, which are products of VLDL and transport cholesterol. As a result, preformed triglycerides are not able to be transported from the intestinal mucosa, leading to the absence of triglycerides in the patient's plasma.

The MTP gene (GenBank X 59657) [39] is composed of the three functional domains, that is: (1) N-terminal domain (codons 22–297), (2) α -helical domain (codons 298–603), and (3) C-terminal domain (codons 604–894). Mutations of the MTP gene [39–42] are: (1) 215 del 1 (exon 2), Q95H (exon 3), I128 T (exon 3), 419 ins 1 (exon 4), Q244E (exon 6), and H297Q (exon 7) at the N-terminal domain, (2) 1147 del 1 (exon 9), 1344 + 5→11 del 7 (intron 10), 1401 ins 1 (exon 11), and 1783C→T (exon 12) at the α -helical domain, and (3) 1867 + 1, G→A (intron 13), 1867 + 5G→A (intron 13), 1989, G→A (exon 14), 2212 del 1 (exon 15), and 2593G→T (exon 18) at the C-terminal domain, respectively.

It is not impossible that defects in other elements involved in the assembly, processing, and secretion of apo B containing lipoproteins may be present in some cases of this disorder, although all published results exhibit defects in the large subunit of MTP in abetalipoproteinemia [3, 43].

In clinical hematology, acanthocytosis is the hallmark of abetalipoproteinemia. Acanthocytes constitute from 50 to 100% of the total red cells in peripheral blood [3]. It is interesting to note that this shape change of acanthocytosis appears to be derived by contact of the red cells with the patient's plasma *in vivo*.

The lipid composition of red cell membranes in abetalipoproteinemia partly reflects the abnormal composition of the plasma lipoproteins [44, 45]. The contents of total phospholipids and cholesterol in the red cell membranes are normal or greater than in normal red cells, and the sphingomyelin:lecithin ratio is increased from 0.9 to over 1.4 [45]. There is a shift to more saturated fatty acids among the sphingomyelin [45]. In our two patients with abetalipoproteinemia, their red cell membrane lipids (μg per 10^{10} red cells, on average) exhibit 1514 for free cholesterol (normal: 1204 ± 103), total phospholipids 2591 (normal: 2604 ± 241), 731 for sphingomyelin (normal: 663 ± 73), 565 for lecithin (normal: 733 ± 64), 479 for phosphatidylserine (normal: 366 ± 38), 793 for phosphatidylethanolamine (normal: 806 ± 86), and 23 for lyso-lecithin (normal: 39 ± 11) (Table 17.5).

The red cells in this disorder appear to gain the acanthocytic shape through an abnormal distribution of lipids between the inner and outer leaflets of the mem-

brane lipid bilayer. However, the exact molecular basis of the acanthocytic shape is not known. It is speculated that it is related to an increase in the surface area of the outer leaflet of the membrane lipid bilayer by incorporation of the extra sphingomyelin relative to the inner leaflet, because sphingomyelin is normally located preferentially in the outer leaflet of the membrane lipid bilayer. This preferential accumulation of sphingomyelin in the outer leaflet may lead to its expansion and the formation of a protrusion of the outer surface of the red cells, i. e., acanthocytosis.

It is surprising to know that red cell membrane lipids in this disorder are maintained quantitatively fairly well, compared with the extremely abnormal lipids in plasma with a striking reduction of the total cholesterol, triglycerides, and total phospholipids. This is because delivery of cholesterol to peripheral red cells appears to be nearly normal even in the absence of VLDL and LDL.

The hematological abnormalities are relatively mild and include mild normocytic anemia with marked acanthocytosis and normal or slightly elevated reticulocyte counts. However, patients may develop more severe anemia due to the nutritional (iron and folate) deficiencies that are associated with fat malabsorption. In fact, in one of our patients with abetalipoproteinemia, there was a moderate hypochromic anemia (red cell counts of $5.51 \times 10^{12} \mu\text{L}^{-1}$, and hematocrit of 31.7%) with the mean corpuscular volume (MCV) of 64 fL with mild reticulocytosis (1.5%), which responded well to iron supplementation. The indirect bilirubin level was 0.4 mg dL^{-1} . The osmotic fragility test demonstrated normal results even after the red cells were incubated at 37°C for 24 h. Sodium transport was not affected, that is: sodium influx: 1.13 mmol L^{-1} red cells per hour (normal: 1.29 ± 0.14), and sodium efflux: 0.21 h^{-1} (normal: 0.24 ± 0.05). Red cell sodium and potassium contents are 20 and 80 mM, respectively. We were able to demonstrate a tremendous increment of Ca^{2+} influx in this patient (440 nmol mL^{-1} red cells per 25 min; normal: $8 \pm 2 \text{ nmol mL}^{-1}$ red cells per 2 h), and increased red cell calcium content ($1.68 \mu\text{g mL}^{-1}$ red cells; normal: 0.69 ± 0.31). Membrane fluidity of the red cells of this disorder appears to be normal (Table 17.6).

To treat patients with abetalipoproteinemia, the gastro-intestinal symptoms respond to restriction of triglycerides containing long chain fatty acids to about 15 g per day. It is evident that tocopherol supplementation does inhibit the progression of the neurological disease and appears to lead to some regression of the symptoms [3]. The retinopathy can be prevented if the supplementation is started early. The myopathy appears to be reversed under this treatment. In our experience on one patient with this disorder, in whom the level of vitamin E was extremely low (less than $0.4 \mu\text{g mL}^{-1}$; normal: 5–20), the intramuscular administration of vitamin E (1 mg kg^{-1} per week) for eight years appeared to prevent the progression of the symptoms, and hematological findings are maintained and not worsened with approximately 50% of acanthocytosis.

Table 17.6 Membrane fluidity evaluated by electron spin resonance (ESR) method in intact red cells of the patients with congenital β -lipoprotein deficiency (acanthocytosis).

Primary lesion	Compensation mechanism	Membrane fluidity (overall)
β -lipoprotein deficiency	(Plasma) $\downarrow\downarrow$ Total cholesterol $\longrightarrow$ (Red cells) Free cholesterol normal $\sim$ increased	Normal
	$\downarrow\downarrow$ Total phospholipids $\longrightarrow$ Phospholipids normal	
	$\downarrow$ % PC $\longrightarrow$ $\downarrow$ % PC	
	$\uparrow$ % SM $\longrightarrow$ $\uparrow$ % SM	
	$\downarrow$ C18: 2 $\longrightarrow$ $\downarrow$ C18: 2	
	$\downarrow$ 24: 1 $\longrightarrow$ $\uparrow$ C24: 1	

PC: phosphatidylcholine, SM: sphingomyelin, PS: phosphatidylserine, and PI: phosphatidylinositol.

17.4

Hereditary High Red Cell Membrane Phosphatidylcholine Hemolytic Anemia (HPCHA)

In 1968, the first case of hereditary nonspherocytic hemolytic disease associated with altered phospholipid composition of the red cells (HPCHA) was described by Jaffé et al. [46]. Since that time, several additional reports have been published [47–50]. This hemolytic disease is characterized by a mild hemolytic anemia with elevated phosphatidylcholine levels in the red cell membranes and normal plasma lipids [46]. The mechanism of the hemolysis has not been described. Similarly, the reason that the accumulation of phosphatidylcholine is confined to the red cell membranes is not known, although the mechanism for that accumulation has been studied extensively by Shohet et al. [51, 52].

We experienced a substantial number of these patients with HPCHA; that is, 31 patients from 19 independent kindred among 1014 cases of hereditary red cell membrane disorders from 605 kindred in the Japanese population over 25 years [35].

Increased phosphatidylcholine affects the morphology and membrane functions of the red cells.

Membrane lipid fluidity is dependent on the type of cholesterol (free or esterified), the class of phospholipids, the molar ratio of cholesterol to phospholipids, the degree of saturation of fatty acids, the length of acyl chains, and the presence or absence of amphipathic compounds, such as lysophosphatides (see Section 2.2.3). Free cholesterol (FC) decreases the membrane lipid fluidity, while esterified cholesterol (EC) increases it. Phosphatidylcholine (PC) increases the membrane fluidity, and sphingomyelin (SM) and phosphatidylethanolamine (PE) decrease it. Short acyl chains and low saturation of fatty acids increase membrane fluidity, and longer acyl chains and high saturation of fatty acids decrease it. Lysophosphatides increase membrane fluidity. Compensation for an alteration in one or more of these variables to maintain normal membrane fluidity has been termed “homeoviscous adaptation” by Sinensky [29]. This phenomenon has been observed in *Escherichia coli*, fungi

(*Fusarium*), *Tetrahymena*, hibernating squirrels, guinea pig lymphatic leukemia cells, and human red cells with lecithin:cholesterol acyl transferase deficiency (Section 17.2). To consider the possibility of a similar “compensatory” mechanism in the red cell membranes of a patient with hereditary high red cell membrane phosphatidylcholine hemolytic anemia, we analyzed the red cell and plasma lipids and also determined the membrane fluidity in the intact cells, as well as in liposomes prepared from all of the red cell lipids along with liposomes prepared from only the red cell phospholipids.

The patient is a 68 year old Japanese woman who was found to have a mild uncompensated hemolytic anemia at age 19 [48].

A marked reticulocytosis ($17.3 \times 10^4 \mu\text{L}^{-1}$) with normocytic (MCV, 103 fL), slightly hyperchromic (MCH, 37.4 pg) anemia (red cell count, $2.62 \times 10^6 \mu\text{L}^{-1}$, hemoglobin, 9.8 g dL^{-1} , hematocrit, 27.1%) was observed. The MCHC was 36.2%. There was slight anisocytosis and mild poikilocytosis with occasional target cells and stomatocytes (Fig. 17.4). Bone marrow aspiration revealed normoblastic erythroid hyperplasia. The WBC count and platelet count were normal.

The serum chemistry examination was normal except for a slight elevation of bilirubin (direct, 0.4 mg dL^{-1} ; indirect, 0.8 mg dL^{-1}). The red cell life span ($^{51}\text{CrT}_{1/2}$) was 16 days. Red cell osmotic fragility was decreased (minimum, 0.40%; maximum, 0.26%). Family studies revealed that her two sons were also affected [48].

The total lipid content, cholesterol, free fatty acids, triglyceride, phospholipids, and β -lipoprotein in plasma were completely normal in the patient [48]. No abnormal lipoprotein fraction was detected.

The contents of free cholesterol and total phospholipids in the patient's red cells (Table 17.7) were $1765 \pm 96 \mu\text{g}$ per 10^{10} red cells (normal: 1202 ± 103) and $3440 \pm 102 \mu\text{g}$ per 10^{10} red cells (normal: 2604 ± 241), respectively [48]. Phosphatidylcholine (PC) ($1242 \pm 59 \mu\text{g}$ per 10^{10} red cells) and sphingomyelin (SM) ($777 \pm 28 \mu\text{g}$ per 10^{10} red cells) levels in the patient's red cells were increased significantly in comparison with control red cells. It should be noted that the ratio of SM to PC (SM/PC) was markedly reduced (0.63 ± 0.03), compared with normal controls (0.90 ± 0.07) [48]. Phosphatidylethanolamine (PE) and phosphatidylserine contents were $843 \pm 35 \mu\text{g}$ per 10^{10} red cells and $468 \pm 17 \mu\text{g}$ per 10^{10} red cells, respectively, indicating that phosphatidylserine (PS) and phosphatidylinositol (PI) levels

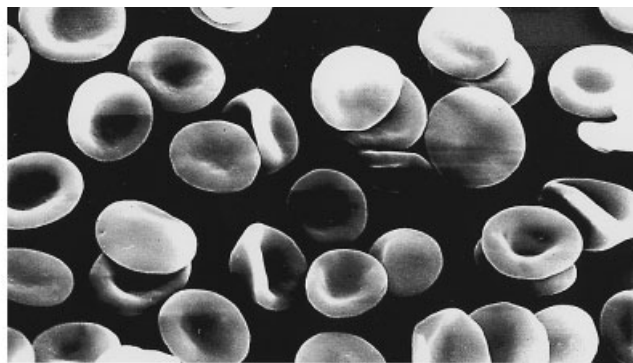


Figure 17.4 Scanning electron micrograph of red cells in hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA).

Table 17.7 Lipids in plasma and red cells in hereditary high red cell membrane phosphatidylcholine hemolytic anemia.**I. Plasma lipids**

	<i>Patient (n = 7)</i>	<i>Normal (n = 20)</i>
Cholesterol (mg dL ⁻¹)	191 ± 25	193 ± 31
Free fatty acids (mg dL ⁻¹)	470 ± 58	450 ± 150
Triglyceride (mg dL ⁻¹)	88 ± 31	95 ± 65
Phospholipids (mg dL ⁻¹)	152 ± 32	180 ± 20
β-Lipoprotein (mg dL ⁻¹)	405 ± 74	350 ± 150
Lipoprotein fraction	Normal	
Phospholipids (%)		
Lysophosphatidylcholine	3.8 ± 2.1	5.5 ± 1.8
Sphingomyelin	15.9 ± 2.0	16.5 ± 2.3
Phosphatidylcholine	72.5 ± 11.1	64.9 ± 12.7
Phosphatidylserine + phosphatidylinositol	2.0 ± 1.5	1.7 ± 1.3
Phosphatidylethanolamine	4.4 ± 4.3	4.4 ± 3.8

In duplicate determinations (mean value ± 1 SD).

II. Red cell membrane lipids

	<i>Patient (n = 4)</i>	<i>Normal (n = 10)</i>
Free cholesterol (FC)	1765 ± 96*	1,202 ± 103
Total phospholipids (PL)	3440 ± 102*	2,604 ± 241
Lysophosphatidylcholine (L-PC)	72 ± 12 (2.1 ± 0.3%)	34 ± 18 (1.3 ± 0.7%)
Phosphatidylcholine (PC)	1242 ± 59* (36.1 ± 1.7%)*	747 ± 73 (28.7 ± 2.8%)
Sphingomyelin (SM)	777 ± 28* (22.6 ± 0.8%)*	674 ± 49 (25.9 ± 1.9%)
Phosphatidylethanolamine (PE)	843 ± 35 (24.5 ± 1.0%)*	805 ± 42 (30.9 ± 1.6%)
Phosphatidylserine (PS) + Phosphatidylinositol (PI)	468 ± 17* (13.6 ± 0.5%)	344 ± 34 (13.2 ± 1.3%)
PC + SM + L-PC/PE + PS + PI	1.59 ± 0.02*	1.27 ± 0.04
FC/PL ratio	1.00 ± 0.02*	0.90 ± 0.04
SM/PC ratio	0.63 ± 0.03*	0.90 ± 0.07

The numbers represent μg per 10¹⁰ red cells. The numbers in parentheses represent the percentage of phospholipids (mean value ± 1 SD).

**P* < 0.01, significant difference.

were substantially increased in absolute amounts but not in the percentages of the total lipids. The ratio of free cholesterol to phospholipids was slightly elevated in the HPCHA red cells (1.00 ± 0.02), compared with controls (0.90 ± 0.04).

Fatty acid composition of the phospholipids revealed that red cell phospholipids were somewhat less unsaturated than normal controls (Table 17.8). The principal

Table 17.8 Fatty acid composition of plasma lipids and red cell lipids of the patient with hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA).

I. Fatty acid composition of plasma lipids	Total lipids		Phosphatidylcholine (PC)		Phosphatidylethanolamine (PE)		Sphingomyelin (SM)	
	HPCHA	Normal	HPCHA	Normal	HPCHA	Normal	HPCHA	Normal
14 : 0	—	—	0.7 ± 0.1	0.7	2.8	1.5	2.7	4.0
16 : 0	27.4%	23.3%	43.7 ± 4.1	41.5	28.1	25.7	34.2	32.0
16 : 1	4.8	2.4	—	—	2.5	1.4	2.4	2.4
18 : 0	8.0	7.6	21.4 ± 0.6	19.6	13.0	17.4	11.1	12.4
18 : 1	23.7	23.4	13.8 ± 1.6	11.9	23.5	21.9	19.2	17.6
18 : 2	16.4	27.7	11.9 ± 1.1	17.6	9.2	11.2	5.3	6.5
20 : 1	0.7	0.8	4.2 ± 0.5	4.0	—	—	—	—
22 : 0	1.3	1.2	—	—	—	—	2.5	3.7
20 : 4	4.3	4.9	—	—	6.2	10.5	1.8	1.8
24 : 0	0.2	1.0	N.D.	—	2.0	1.6	3.7	2.8
24 : 1	0.6	0.6	—	—	1.6	3.9	5.0	5.5
22 : 6	3.8	2.5	—	—	—	—	—	—

Minor components less than 2% are not included (20 : 0, 20 : 5, 22 : 5).
In duplicate determinations, except for those of phosphatidylcholine in HPCA (*) in triplicates.

II. Fatty acid composition of red cell lipids

	Total lipids		Phosphatidylcholine (PC)		Sphingomyelin (SM)		Phosphatidylethanol-amine (PE)		Phosphatidylserine (PS)	
	HPCHA (n = 3)	Normal (n = 7)	HPCHA (n = 3)	Normal (n = 7)	HPCHA (n = 3)	Normal (n = 7)	HPCHA (n = 3)	Normal (n = 7)	HPCHA (n = 3)	Normal (n = 7)
DMA	1.4	0.5 ± 0.4	—	—	—	—	5.4	4.9 ± 0.4	—	—
16 : 0	21.8	21.4 ± 1.2	36.7	35.4 ± 1.7	30.1*	25.1 ± 1.9	20.1*	17.4 ± 2.0	3.7	3.5 ± 0.7
DMA	1.2	2.0 ± 0.5	—	—	—	—	5.8	7.3 ± 1.1	—	—
18 : 0	15.7*	14.5 ± 0.4	13.3*	12.4 ± 0.5	6.2*	7.0 ± 0.5	10.7*	8.7 ± 0.2	49.3	47.8 ± 2.8
18 : 1	15.1*	13.3 ± 0.9	23.3*	17.0 ± 0.7	1.8	2.6 ± 1.1	17.0	17.1 ± 0.7	5.7*	7.9 ± 1.0
18 : 2	8.0*	10.8 ± 1.1	11.7*	21.9 ± 1.3	1.6	2.7 ± 1.3	3.8*	6.4 ± 1.2	1.7	2.6 ± 0.6
20 : 0	—	—	—	—	1.6	1.8 ± 0.4	—	—	—	—
20 : 3	0.9	1.2 ± 0.2	1.2	1.4 ± 0.4	—	—	—	—	1.4	1.6 ± 0.2
20 : 4	9.9*	12.4 ± 0.9	4.0	4.4 ± 0.5	—	—	14.0*	16.2 ± 0.9	15.9*	18.0 ± 1.0
20 : 5	1.8	1.4 ± 0.4	1.8	1.6 ± 0.4	—	—	2.7	2.9 ± 0.8	—	—
22 : 0	—	—	—	—	8.0	7.4 ± 0.7	—	—	—	—
22 : 4	—	—	—	—	—	—	3.9	3.6 ± 0.4	2.2	2.1 ± 0.4
22 : 5	3.2	2.8 ± 0.2	—	—	2.5*	4.1 ± 0.7	4.4*	3.5 ± 0.6	3.8*	2.8 ± 0.3
22 : 6	8.3	7.4 ± 0.9	3.9*	2.6 ± 0.5	—	—	9.0	8.9 ± 1.0	13.0*	10.6 ± 0.9
24 : 0	4.2	4.9 ± 0.6	—	—	20.1	19.5 ± 0.9	—	—	—	—
24 : 1	3.9	4.1 ± 0.7	—	—	24.7	26.4 ± 2.9	—	—	—	—
Unsaturations (%)	54.2	55.2 ± 0.9	48.7*	51.3 ± 1.1	34.7*	37.9 ± 1.6	56.9*	60.6 ± 1.3	45.6	47.7 ± 2.3
Unsaturations index	159.7	165.9 ± 5.9	106.0*	114.9 ± 6.7	53.3*	63.9 ± 3.9	198.7	204.9 ± 13.1	196.0	192.1 ± 6.8

The numbers represent the percentage of phospholipid.

*P < 0.01, significant difference.

changes seen were moderate reductions of arachidonic and linoleic acids and increases of stearic acid in the patient's red cells [48].

Electron spin resonance studies on membrane lipid fluidity were carried out over a wide range of temperatures (15 °C to approximately 47 °C) [48] (Table 17.9). The order parameter (*S*) was calculated from the ESR spectra in the intact red cells, in the total lipid (cholesterol-containing) liposomes, and in the pure phospholipid (cholesterol-depleted) liposomes.

In the liposomes of the pure phospholipids of the patient's red cells, the order parameters were lower than those from normal controls. Thus, pure phospholipid liposomes in the HPCA red cells showed appreciably increased fluidity [48]. In contrast, in the liposomes of total lipids containing free cholesterol prepared from the patient's red cells, the order parameters were slightly lower than those from normal controls. The differences, however, were not statistically significant.

In the intact red cells, the order parameters of ESR spectra in the HPCA patient also appeared to be lower than those in normal controls (Fig. 17.5). The differences, however, were minimal and not statistically significant [48].

Phospholipids and cholesterol are the major lipids in human red cell membranes. The interactions between phospholipids and cholesterol impose a degree of immobility on the portion of the lipid molecules for some distance in the lipid bilayer of the red cell membranes.

The addition of cholesterol to pure phospholipid membranes decreases fluidity and increases the order parameters measured by ESR. Fluidity in red cell membranes studied with hydrophobic probes (such as SAL) can be detected by electron spin resonance. Membrane fluidity (see Section 2.2.3) is influenced by: (1) the amount of free cholesterol; (2) the nature of the phospholipids; (3) the degree of fatty acid saturation; and (4) the length of the acyl chains.

A "compensatory" mechanism for abnormal membrane fluidity has been observed in general biology [29] and also in one example of a hemolytic condition in human red cells of lecithin:cholesterol acyl transferase deficiency [25].

The possibility of a similar mechanism was investigated in the red cells of a patient with hereditary high red cell membrane phosphatidylcholine hemolytic anemia, in which phosphatidylcholine is increased only in the red cell membranes.

The fluidity of pure phospholipid liposomes prepared from the patient's red cells was increased significantly, chiefly because of the marked increase of phosphatidylcholine, which is intrinsic to the disorder [48]. A change in the phospholipids, such as in the class of phospholipids, in fatty acid composition, in saturation/unsaturation, and in carbon number of acyl chains, appeared to compensate only minimally for the marked increase in the fluidity caused by the increased phosphatidylcholine.

In contrast, the cholesterol-containing "total liposomes" showed virtually normal fluidity, apparently because of the presence of free cholesterol, which made the membranes less fluid. Thus, the increased membrane fluidity induced by the increased PC was essentially corrected by the increased cholesterol [48].

Table 17.9 Membrane fluidity by electron spin resonance (ESR) evaluated by order parameters in intact red cells, total lipid liposomes containing free cholesterol, and phospholipid (free cholesterol-depleted) liposomes in red cells of the patient with hereditary high red cell membrane phosphatidylcholine hemolytic anemia.

Tem- pera- ture	Sample	Order parameter (S)				
		Normal (n = 8)	P	HPCHA (n = 5) Δ	P	
37 °C	Intact red cells	0.660 ± 0.005	— NS —	0.657 ± 0.006	0.003 ± 0.002	NS † *
	Total lipid (FC +) liposomes	0.648 ± 0.003	— NS —	0.643 ± 0.004	0.005 ± 0.002	
	Phospholipid (FC−) liposomes	0.606 ± 0.003	— † —	0.593 ± 0.005	0.013 ± 0.003	
43 °C	Intact red cells	0.641 ± 0.004	— NS —	0.636 ± 0.006	0.005 ± 0.003	NS † *
	Total lipid (FC +) liposomes	0.616 ± 0.004	— † —	0.609 ± 0.005	0.007 ± 0.002	
	Phospholipid (FC −) liposomes	0.581 ± 0.003	— * —	0.565 ± 0.005	0.016 ± 0.002	
31 °C	Intact red cells	0.690 ± 0.006	— NS —	0.682 ± 0.007	0.008 ± 0.002	NS † *
	Total lipid (FC +) liposomes	0.681 ± 0.006	— NS —	0.672 ± 0.004	0.009 ± 0.002	
	Phospholipid (FC −) liposomes	0.634 ± 0.005	— * —	0.617 ± 0.005	0.017 ± 0.003	

FC, free cholesterol; HPCHA, hereditary high red cell membrane phosphatidylcholine hemolytic anemia.

*Significant, $P < 0.01$. **Significant, $P < 0.02$. (†); NS, not significant.

Mean value ± SD.

Δ, Differences of order parameters between normal and HPCHA.

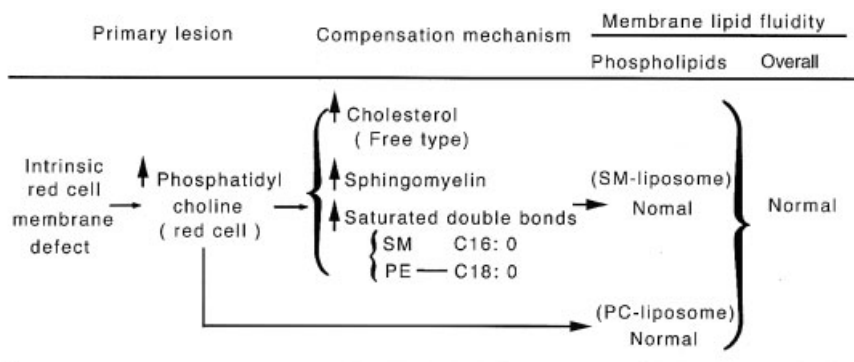


Figure 17.5 Red cell membrane lipid fluidity compensated by alterations of lipid composition in hereditary high red cell membrane phosphatidylcholine hemolytic anemia (HPCHA).

Fluidity was also examined in the intact red cells of the HPCA patient. Nearly normal membrane fluidity was found in the whole cells. Thus, the overall membrane fluidity was maintained nearly normally in the intact red cells by the increased cholesterol, even with the increased phosphatidylcholine. This suggests that in the intact cells, also, the elevated cholesterol seems to compensate for fluidity abnormalities that might otherwise occur because of the elevated PC. Therapeutically, splenectomy in this disorder is not advisable, because no beneficial effect has been observed [49].

17.5

α -Lipoprotein Deficiency (Tangier Disease)

Tangier disease is a rare inherited disorder which is characterized by a severe deficiency or absence of normal high density lipoproteins (HDL) in plasma and by the accumulation of cholesteryl esters in many tissues including tonsils, peripheral nerves, intestinal mucosa, spleen, liver, bone marrow, lymph nodes, thymus, skin, and cornea [4]. The finding of virtual HDL deficiency, a low plasma cholesterol concentration associated with normal or elevated triglyceride levels in subjects with hyperplastic orange-yellow tonsils and adenoidal tissue is pathognomonic for the condition. The plasma concentration of apolipoprotein (apo) A-I is extremely low (lower than 3 % that of controls). Heterozygous individuals exhibit no clinical symptoms but half-normal levels of HDL cholesterol and apo A-I.

Tangier disease was first discovered in 1961 in the two probands who lived on the Tangier island in the Chesapeake Bay, Virginia, USA [53]. Since that time, this disease has been diagnosed in about 70 patients from 60 families [4].

The symptoms of Tangier disease patients result from cholesteryl ester deposition in many organs, which are frequently part of the reticuloendothelial system. Cells other than histiocytes accumulating cholesteryl esters in Tangier disease include fibroblasts of the cornea, melanocytes, Schwann cells, neurons, and nonvascular smooth muscle cells. The most characteristic features are histiocytic manifestations. Tangier histiocytes appear as foam cells that contain sudanophilic lipid droplets and occasionally as crystalline material. The droplets within the cytoplasm are mostly not bound by membranes, and consist of deposits of cholesteryl oleate outside of the lysosomes [4].

The bone marrow contained numerous foam cells full of lipid droplets in the cytoplasm. Splenomegaly is accompanied by mild thrombocytopenia [54]. The cells in the spleen were filled with intracytoplasmic lipid droplets and scattered clusters of cholesterol crystals. Lymphadenopathy is not clinically apparent, but both normal sized and enlarged lymph nodes exhibit bright-yellow streaks and morphologic characteristics similar to those present in the tonsils, and the cholesteryl ester content is increased by a factor of 100 compared with control lymph nodes. Hepatic parenchymal cells are usually not infiltrated with lipids. Liver functions are usually normal. The gross appearance of the rectal mucosa is abnormal in every case examined and may be the most reliable physical finding.

Biopsy of the rectal mucosa or colonic polyps reveals foamy histiocytes throughout the mucosa and submucosa.

The most frequent anomaly of peripheral blood in this disorder is thrombocytopenia with platelet counts ranging between 30 000 and 120 000 cells per microliter. Hemolysis and hemolytic anemia have been reported with stomatocytosis [55–57], which may be secondary to HDL deficiency. The altered red cell morphology may be explained by the decrease of cholesterol and increase of phosphatidylcholine in the membranes of Tangier red cells [4]. The osmotic resistance was decreased probably due to the reduced cholesterol/phosphatidylcholine ratio in Tangier red cells. The anion transport by band 3 was also impaired. Sodium and potassium fluxes were normal.

Homozygotes of this Tangier disease exhibit a low plasma concentration of total cholesterol. The percentage of esterified cholesterol is normal. Triglycerides are frequently elevated to 300 to 400 mg dL⁻¹. Plasma phospholipid concentrations are decreased to 30 to 50 % of normal values. The sphingomyelin to phosphatidylcholine ratio is decreased. The most characteristic feature is the absence of α -migrating lipoproteins, as well as the loss of a distinct pre- β band and the presence of a diffuse β -pre- β band, confirming the virtual absence of HDL [4]. The gross lipid and lipoprotein compositions of the chylomicrons are normal. The plasma concentration of HDL-C in Tangier homozygotes is reduced to essentially zero due to the absence of mature α -HDL [58]. The exclusive presence of apo A-I in pre- β -HDL together with the failure of Tangier plasma to convert exogenous apo A-I into α -HDL are specific biochemical markers which distinguish Tangier disease from other familial HDL deficiency syndromes.

Regarding apolipoproteins, the plasma concentration of apo A-I is markedly decreased to approximately 1 to 3 % of normal [4]. Human apo A-I is synthesized in enterocytes and hepatocytes as a 267 amino acid long preproapolipoprotein (pre-proapo A-I) containing an 18 amino acid peptide and a six amino acid propeptide [59–61]. The plasma concentration of apo A-I is also severely reduced to 5 to 10 % of normal.

As for the pathophysiology of Tangier disease, HDL deficiency and cholesterol accumulation in macrophages of this disorder clearly indicate that the adenosine triphosphate binding cassette transporter 1 (ABC 1) plays a key role in the formation of HDL and in the regulation of cholesterol homeostasis in macrophages [62–65], which will be discussed later. However, the exact mechanism is still not known. ABC 1 may form a channel within the plasma membrane through which phospholipids (sphingomyelin, phosphatidylserine, and phosphatidylinositol) and cholesterol are transferred from the inner leaflet to the outer leaflet of the plasma bilayer membranes, as a sort of flopper [66, 67]. In exchange, phospholipids, which are more abundant in the outer leaflet (especially, phosphatidylcholine), may be transferred from the outer leaflet to the inner leaflet, by ABC 1 as a flipper [67]. ABC 1 may not only translocate lipids between the two leaflets of the plasma membrane, but also may serve as a protein component of vesicles or rafts that target lipids and proteins between lipid-rich intracellular lysosomes and the plasma membrane. For example, the multidrug resistance proteins

(MDRs) 1 and 3 are important for the trafficking of proteins and lipids between intracellular organelles and the plasma membrane [68]. Thus, both the floppase/flippase model and the trafficking model may explain the defective lipid efflux. Whatever the exact mechanism is, defective lipid efflux from ABC 1 deficient Tangier cells appears to interfere with the formation of lipid rich HDL.

The clinical phenotype of Tangier disease is inherited as an autosomal recessive trait, and the biochemical phenotype is inherited as an autosomal codominant trait. Genetically, the Tangier locus (*HDLDT* 1; HDL-deficiency Tangier type 1) was mapped to 9q31 [69]. The adenosine triphosphate (ATP) binding cassette transporter (ABC 1) is identified as the defective gene in Tangier disease [62–65]. Mutations on this gene have been reported at least in 13 families with this disorder, that is: (1) Gln537Arg, (2) G 1764 del (frameshift mutation), (3) Cys1417Arg (abnormal splicing), (4) deletion of exons 39 to 48 (truncation), (5) Asn875Ser, (6) Ala877Val, Val339Arg, and Ile823Met, (7) Ala877Val, and W530Ser, (8) 110 base pair Alu sequences replace 14 base pairs in exon 12, (9) Asn875Ser, (10) two base pairs deletion in exon 22 (frameshift), (11) one base pair insertion in exon 22 (frameshift), (12) 14 base pairs insertion following nt 5697 (frameshift), and 138 base pairs insertion following nt 5062, and (13) Arg527Trp (deletion of several exons suspected).

The human ABC 1 gene on chromosome 9q31 encompasses 49 exons within more than 70 kb of genomic sequence [70]. The cDNA encodes for 2201 amino acids [71]. The ATP binding cassettes are formed by amino acid residues 866 to 1047 and 1879 to 2060, respectively. Each of the two transmembrane domains consists of six transmembrane-spanning segments. The two domains are linked by a hydrophobic segment. The transmembrane segments appear to form the wall of an aqueous chamber within the plasma membrane. This chamber is opened to the extracellular space and to the lipid face of the plasma membrane, but not to the cytosol. Substrates of ABC 1 may be transported through this channel.

There is no specific treatment for Tangier disease [4]. Drugs known to increase HDL levels in normal subjects are ineffective in this disorder. Generally speaking, patients with Tangier disease have very low levels of LDL cholesterol, so hypercholesterolemia is rarely a cardiovascular risk factor in Tangier disease. Instead, hypertriglyceridemia is an independent risk factor for myocardial infarction, which is frequently observed in this disorder. Dietary intervention with reduction of intake of total fat and especially saturated fatty acids is effective in lowering triglycerides in Tangier disease.

17.6

Abnormalities Associated With Other Diseases (Target Cells and Spur Cells)

In patients with liver disease, the anemia is of complex etiology [1, 2]. Common causes of the anemia are blood loss by bleeding, hypersplenism, iron deficiency, folate deficiency, and bone marrow suppression from excessive alcohol ingestion, viral hepatitis, and other factors.

There are two major morphological abnormalities in red cells of the patients with these hepatic disorders; i. e., target cells, and spur cells (see Section 13.5). Target cells are associated mainly with obstructive liver disease, whereas spur cells are associated mainly with hepatocellular disease. These red cell abnormalities are not only present separately, but also are occasionally observed together in clinical practice. Therefore, the situation is fairly complicated, because the strict differentiation of one from the other appears to be difficult depending on the clinical picture of these liver diseases.

The extent of anemia in liver diseases with spur cells is definitely more serious than that with target cells, that is: in the former condition in six patients with spur cells, hematocrit is $25.4 \pm 4.5\%$, mean corpuscular volume (MCV) 99.8 ± 11.7 fL, reticulocytes $5.8 \pm 5.3\%$, and total bilirubin 12.5 ± 7.1 mg dL⁻¹, in contrast to the latter condition in 15 patients with target cells, hematocrit $33.2 \pm 5.8\%$, MCV 99.6 ± 11.8 fL, reticulocytes $3.9 \pm 1.9\%$, and total bilirubin 7.3 ± 4.8 mg dL⁻¹, compared with those in a normal control, hematocrit $44.1 \pm 2.3\%$, MCV 89.7 ± 2.6 fL, reticulocytes $1.0 \pm 0.2\%$, and total bilirubin 0.7 ± 0.2 mg dL⁻¹, respectively.

Our studies on 222 cases of various liver diseases indicate that 137 cases (62%) demonstrate an increased diameter of peripheral red cells, including 81 cases with thin macrocytosis, 39 cases with target macrocytosis, and 17 cases with thick macrocytosis.

Bone marrow in these patients with liver diseases is usually normocellular to hypercellular with the presence of macronormoblasts that differ from megaloblasts. Apparent red cell life span ($^{51}\text{CrT}_{1/2}$) is shortened; that is, less than 24 days in 48 cases out of a total of 68 cases (approximately 70%), 20 days on average, and less than 16 days in 14 cases. Therefore, the results indicate that the red cell life span is definitely shortened, but not strikingly so.

The most frequent complication of liver diseases is red cell membrane abnormalities with target cells. The primary lesions are the cirrhosis of the liver, obstructive jaundice, hepatitis, and others. In target cells, red cell membrane lipid abnormalities are increased free cholesterol and phosphatidylcholine. In our studies on red cell membrane lipid analyses in 15 patients with liver diseases accompanying target cells, free cholesterol is 1755 ± 189 μg per 10^{10} red cells (normal: 1202 ± 103), and total phospholipids 3127 ± 389 μg per 10^{10} red cells (normal: 2657 ± 207). The compositions of each fraction of the phospholipids in these patients are phosphatidylcholine 1357 ± 157 μg per 10^{10} red cells (normal: 883 ± 29), sphingomyelin 745 ± 92 (normal: 748 ± 60), phosphatidylethanolamine 757 ± 60 (normal: 785 ± 23), phosphatidylserine 231 ± 54 (normal: 207 ± 71), and lyso-phosphatidylcholine 37 ± 26 (normal: 34 ± 21). The marked increments of free cholesterol and phosphatidylcholine, which are mainly located at the outer leaflet of the red cell membrane lipid bilayer, increase the surface area at the outer leaflet, although the content of intracellular hemoglobin is basically unchanged. This mechanism is most feasible for producing target cells in some liver diseases. Target cells will disappear reversibly, when the lipid abnormalities in plasma and red cell are normalized.

An acquired defect of the enzymatic activity of lecithin:cholesterol acyltransferase (LCAT) can be observed in liver diseases [1, 2]. In this situation, the content of free cholesterol is increased and that of esterified cholesterol is decreased in the patient's plasma. In the red cells, the increased free cholesterol and phosphatidylcholine are expected. There is no direct quantitative correlation between the degree of decreased enzymatic activities of LCAT and that of abnormal red cell lipids.

In liver diseases with target cells, the degree of hemolysis appears to be relatively mild, probably because the surface area of red cell membranes is increased in target cells, and also the pitting function by the spleen of normal size is not excessive [1, 2].

In contrast, patients of spur cell anemia exhibit moderately severe hemolysis, marked indirect hyperbilirubinemia, splenomegaly, and severe hepatic dysfunctions, as shown above (see Section 13.5). The level of spur cells in peripheral blood is more than 20% of the total red cells, which can be confirmed on a wet film under light microscopy with the dark field apparatus. The red cell life span of spur cells is distinctly shortened probably because of splenic sequestration in the presence of splenomegaly. The patients with spur cell anemia exhibit moderate to severe anemia, increased reticulocytosis, extensive jaundice, splenomegaly, ascites, and hepatic encephalopathy.

Spur cell anemia can be produced mainly due to acquired abnormalities of red cell lipids (see Section 13.5). Free cholesterol is markedly increased concomitant to the nearly normal level of total phospholipid content in the red cells. Thus, a molar ratio of free cholesterol to total phospholipids in the red cell membrane is clearly increased, such as 1.60 compared with 0.95 in normal subjects. In our studies on red cell membrane lipid analyses in six patients with spur cell anemia, free cholesterol is $1917 \pm 153 \mu\text{g}$ per 10^{10} red cells (normal: 1202 ± 103), and total phospholipids $2799 \pm 282 \mu\text{g}$ per 10^{10} red cells (normal: 2657 ± 204). The composition of each phospholipid fraction is $1088 \pm 54 \mu\text{g}$ per 10^{10} red cells (normal: 883 ± 29) of phosphatidylcholine, 716 ± 106 (normal: 748 ± 60) of sphingomyelin, 713 ± 53 (normal: 785 ± 23) of phosphatidylethanolamine, 245 ± 52 (normal: 207 ± 71) of phosphatidylserine, and 37 ± 21 (normal: 34 ± 21) of lyso-phosphatidylcholine, respectively. An enormous amount of free cholesterol appears to be transferred from plasma to red cell membranes due to the increased free cholesterol/total phospholipid ratio in low density lipoproteins, which are laden with excessive cholesterol and are deficient of apolipoprotein A-II.

The cholesterol-laden, incipient spur cell appears to be detained and conditioned by the spleen. Cholesterol may affect the function of band 3 by influencing band 3 oligomerization or its interaction with other membrane proteins.

Acanthocytosis due to the deficiency of β -lipoproteins (see Section 13.2) is similar to the spur cell anemia in red cell morphology. However, the composition of abnormal red cell membrane lipids is qualitatively different in these two conditions. The free cholesterol/total phospholipid ratio is essentially normal in acanthocytosis with decreased phosphatidylcholine content concomitant to increased sphingomyelin in the red cell membranes, contrary to spur cell anemia, in which, in addition to

strikingly increased free cholesterol and total phospholipids, a marked increase of phosphatidylcholine is accompanied by a normal sphingomyelin content, leading to the strikingly elevated free cholesterol/total phospholipid ratio. The shortening of red cell survival is only mild in acanthocytosis. The main reason why spur cell anemia demonstrates severe hemolytic anemia appears to be due to the presence of substantial splenomegaly with portal hypertension, and splenic congestion, which are not observed in acanthocytosis.

One should be aware of alcohol-induced abnormalities for proerythroblasts in the bone marrow [1, 2]. In the presence of these abnormalities, the overall clinical picture becomes extremely severe, because excessive intake of alcohol induces the degenerative vacuoles in the cytoplasm of erythroblasts, especially of proerythroblasts more than of mature erythroblasts in the bone marrow. The level of peroral alcohol intake to produce the abnormalities appears to be 200–400 g of ethanol per day for 5–7 consecutive days, which can induce a definite vacuole formation in the cytoplasm of proerythroblasts lasting for 12–14 days.

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Closing remarks

The genome exists basically in the nucleus, and its expression is controlled by many factors including promoter functions and epigenetic control mechanisms; methylation, phosphorylation, acetylation, chromatin packaging, and so on.

In general, the genome is expressed in the nucleus, and genomic messages are transferred via mRNA into Golgi's apparatus in the cytoplasm to produce the determined proteins. The proteins produced in the cytoplasm are carried by presently unknown trafficking transport proteins to the cell membranes. The proteins transferred near the cell membranes should be incorporated precisely into the determined positions of the stereotactic ultrastructure of the cell membranes. The proteins, which are placed in order in the cell membranes, are only allowed to express their cellular functions (Fig. 18.1).

With regard to these processes from genotypes to phenotypes, we have actually studied the genomic mutations of determined red cell membrane protein genes and the protein contents in the cell membranes. However, only some stages of these steps have been clarified. Postgenomic investigations will be critical to the

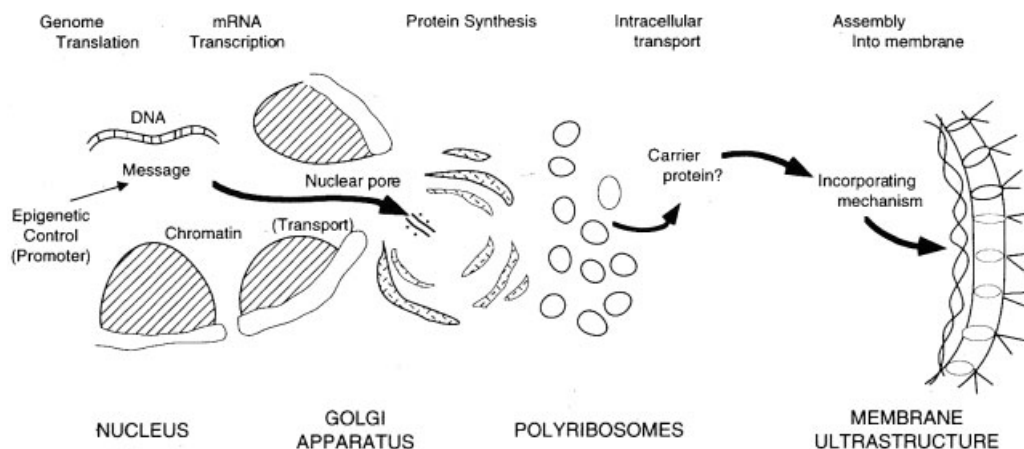


Figure 18-1 Schematic diagram of genetic control for morphogenesis in a red cell membrane from genetic message to membrane proteins in the membrane *in situ*.

elucidation of the pathogenesis of red cell membrane disorders in the future. Clarification of the intracellular trafficking mechanism of membrane proteins in the cytoplasm should be one of the crucial topics.

As for cell-to-cell contact and transduction of cellular message, adhesion molecules and blood group antigens (expressed or cryptic) on the cell surface should play their important role. In addition, the membrane lipid macrodomain and microdomain, especially membrane lipid rafts, will become one of the major targets to investigate a precise mechanism of intracellular protein–lipid interactions in red cell membrane in normal and disease states.

The exact mechanisms of membrane morphogenesis and of the formation of red cell shape have still not been clarified. This problem has been a significant stumbling block to elucidation of the pathogenesis of red cell membrane disorders, especially hereditary spherocytosis, hereditary elliptocytosis, and hereditary stomatocytosis, in which abnormal red cell shapes, such as those in microspherocytosis, elliptocytosis, and stomatocytosis are hallmarks of these disorders.

In human beings, the role of the spleen, which determines the survival of red cells, normal and abnormal, should be critically considered.

The enucleation event at the final stage of erythroblasts is still a mystery.

These processes are totally independent of the genomic states. Therefore, genotype is obviously only one of the definite determinants for phenotypic expressions. We are now at the dawn of the era of postgenomic investigations.

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